AD_____

Award Number: W81XWH-11-1-0570

TITLE: Fatty Acid Synthase Activity as a Target for c-Met Driven Prostate Cancer

PRINCIPAL INVESTIGATOR: David T. Coleman

CONTRACTING ORGANIZATION: Louisiana State University Health Sciences Center
Shreveport, LA 71130

REPORT DATE: July 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual Summary	1 July 2011 - 30 June 2013

4. TITLE AND SUBTITLE
Fatty Acid Synthase Activity as a Target for c-Met-Driven Prostate Cancer.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0570

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David T. Coleman

E-Mail: dcole1@lsuhsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Louisiana State University Health Sciences Center

Shreveport, LA 71130-3932

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer (PCa) is the most common noncutaneous neoplasia diagnosed in men and represents roughly 10% of cancer-associated mortalities. The lethal phenotype of PCa is primarily characterized by progression of tumor cells to castration-resistance and metastasis. Organ-confined PCa is curable with surgery and/or radiation therapy, however, as the disease becomes metastatic, the likelihood of survival becomes minimal. Key influences in the progression to metastasis are growth factor receptors including the receptor tyrosine kinase c-Met. During this research period, I have acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation regulates its stability. Inhibition of palmitoylation reduces the expression of c-Met in prostate cancer cell lines. This protein loss occurs post-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies and confocal microscopy, we determined that inhibition of palmitoylation reduces the stability of newly synthesized c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol suggest c-Met itself is palmitoylated. Observing palmitoylation kinetics has provided evidence that c-Met is palmitoylated in the ER prior to cleavage of the c-Met precursor. Mass spectrometry and site-directed mutagenesis are currently being performed to identify the specific cysteine residue/s that are palmitoylated to aid future studies. Identification is a highly significant finding with regard to the potential for future therapeutic development targeted at restricting the function of c-Met in prostate cancer.Prostate cancer PCa) is the most common noncutaneous neoplasia diagnosed in men and represents roughly 10% of cancer-associated ortalities. The lethal phenotype of PCa is primarily characterized by progression of tumor cells to castration-resistance and metastasis. rgan-confined PCa is curable with surgery and/or radiation therapy, however, as the disease becomes metastatic, the likelihood of survival comes minimal. Key influences in the progression to metastasis are growth factor receptors including the receptor tyrosine kinase c-Met. uring this research period, I have acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation egulates its stability. I nhibition of palmitoylation reduces the expression of c-Met in prostate cancer cell lines. This protein loss occursst-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization athways, as well as surface biotinylation studies and confocal microscopy, we determined that inhibition of palmitoylation reduces the stability ewly synthesized c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a k-chemistry based palmitate-labeling protocol suggest c-Met itself is palmitoylated. Observing p almitoylation kinetics has provided evidence tt c-Met is palmitoylated in the ER prior to cleavage of the c-Met precursor. .

15. SUBJECT TERMS
prostate cancer, c-Met, palmitoylation, fatty acid synthaseprostate cancer, c-Met, palmitoylation, fatty acid synthase

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	223737	

Table of Contents

Fatty Acid Synthase Activity as a Target for c-Met Driven Prostate Cancer
Grant Number: PC102179
Final Report for Early Termination: 01 July 2011 – 30 March 2013

Introduction

The overarching goal of this project is to understand the mechanism by which fatty acid synthase (FASN) activity regulates the expression levels of the oncogenic c-met receptor tyrosine kinase in prostate cancer. My earlier work identified a connection between FASN activity and c-Met protein expression; and more recent data from this reporting period strongly suggests FASN-derived palmitate is required as a post-translation modification to c-Met in order to maintain its stability and trafficking to the plasma membrane. Conclusive evidence for this regulatory mechanism would implicate fatty acid synthase activity and/or palmitoylation as unique therapeutic targets for reducing c-Met expression.

The Specific Aims for this project include:
Aim 1: To determine 1) if c-Met is itself palmitoylated, and if so, at which cysteine residues and 2) if this modification is required for trafficking of c-Met
Aim 2: To investigate a possible correlation of fatty acid synthase with c-Met expression in prostate cancer tissue samples and to investigate a possible causal link between FASN overexpression and c-Met driven prostate cancer using *in vivo* animal models

Body

The work detailed in this project is a continuation of initial findings that determined inhibition of FASN activity reduced c-Met protein levels. Given that c-Met is an important promoter of prostate cancer metastasis, we sought to elucidate the mechanism by which FASN activity maintains c-Met expression. Our preliminary findings suggest palmitoylation, either of c-Met itself or of other proteins regulating its stability, is involved. In this project, I have detailed experiments to determine if c-Met is palmitoylated and how palmitoylation regulates its stability. Through the course of this funded proposal I have made significant progress on the experiments proposed in Aim 1, and designed the tools required to address the experiments detailed in Aim 2 as will be discussed below. In addition, I have obtained the training and research experience required for the PhD degree.

Determined c-Met to be a palmitoylated protein

I have used both an acyl-biotin exchange technique (data not shown) and a click-chemistry based palmitate-labeling protocol to provide convincing evidence that c-Met is actually palmitoylated (Fig. 1A, B, and C). This evidence has been established in multiple cell lines including DU145 and PC3 prostate cancer cell lines. The binding of labeled palmitate (Az-Palm) to c-Met is sensitive to reducing agents (NH2OH) indicating the linkage is via a thioester bond at a cysteine residue (Fig. 1A). In addition, labeling with an equal but alternate palmitate, alkyne-palmitate (ODYA), can be prevented in the presence of the palmitoylation inhibitor 2-bromopalmitate (2BP) at time points prior to loss of significant c-Met protein which supports the specificity of labeling (Fig. 1B). I

was further able to determine that c-Met is palmitoylated upon synthesis in its 170kd precursor form, most likely in the endoplasmic reticulum (ER), and then remains palmitoylated as it is cleaved to the mature 140kd form in the Golgi prior to being trafficked to the plasma membrane. Using cycloheximide (CX) to inhibit protein synthesis, brefeldin A (BF) to inhibit transport of protein from the ER to the cis-Golgi, or monensin (MN) to inhibit protein transport from the trans-Golgi to the plasma membrane; we were able to determine that only newly synthesized c-Met becomes labeled, that the 170kd precursor becomes labeled prior to entering the cis-Golgi, and that palmitoylation is maintained without reaching the plasma membrane (Fig. 1C). We plan to perform additional experiments to determine if ligand activation modifies the kinetics of c-Met palmitoylation. This report would be the first evidence for c-Met palmitoylation or palmitoylation of any oncogenic receptor tyrosine kinase. Palmitoylation is known to regulate the activity of a number of other kinases and GTPases that are major influences on prostate cancer growth and metastasis. Samples are currently being analyzed by mass spectrometry at University of South Alabama proteomics facility to conclusively identify the cysteine residue/s linked to palmitate. Future work within this subaim will rely on the information provided by the mass spectrometry analysis. The results will allow me to make mutations at the palmitoylated cysteine residue/s in order to examine the specific effects of c-Met palmitoylation on prostate cancer growth, invasion, and metastasis rather than being limited to the use of general palmitoylation inhibitors.

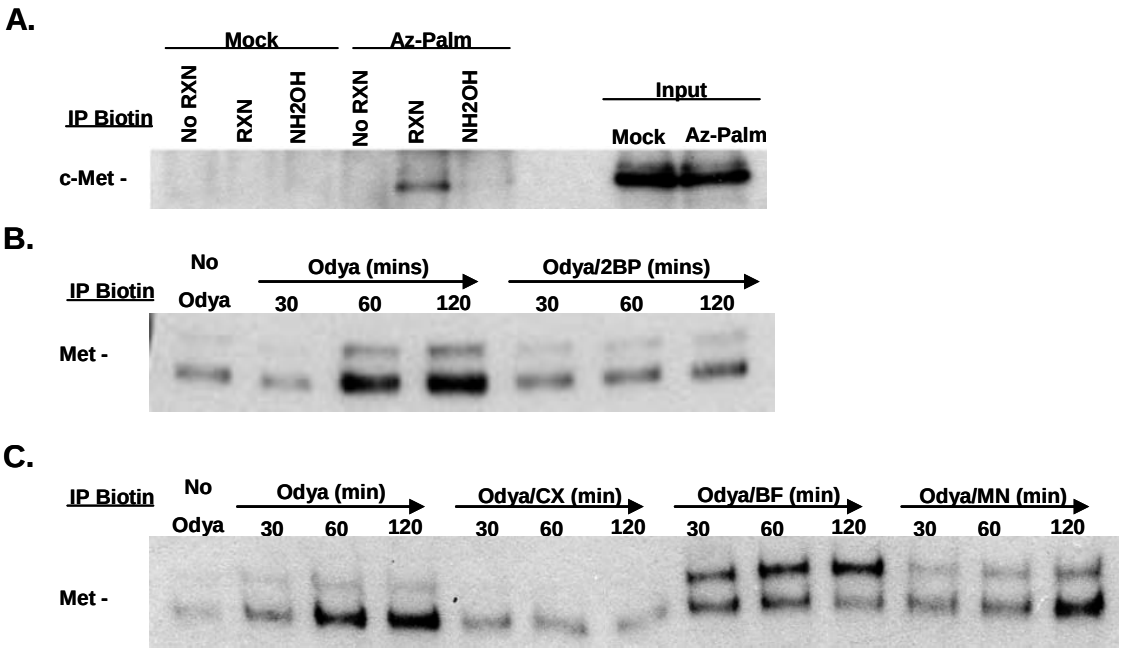


Figure 1. Newly synthesized c-Met is palmitoylated in the Golgi in its 170kd precursor form. For each panel, a Click-chemistry-based technique was used to biotin label and immunoprecipitate palmitoylated protein. A.) DU145 prostate cancer cells were incubated with azide-linked palmitate or mock control for 5 hours. Equal protein for each set was separated for a Click reaction (RXN) or not (No RXN) to link biotin to palmitate having been reduced (NH2OH) to remove palmitate prior to the reaction as a control or not (RXN). Identification of c-Met as a palmitoylated protein is shown by Western blotting. Relative input levels for mock and the labeled sample are shown. B.) DU145 cells were incubated with an alkyne-linked palmitate (Odya) or without (No Odya) for the indicated times in the presence or absence of 100 µM 2BP. Levels of palmitoylated c-Met are demonstrated by Western blotting. C.) As above, DU145 cells were labeled with alkyne-linked palmitate (Odya) or without (No Odya) for the indicated times in the presence of cycloheximide (10 µg/ml) (CX), brefeldin A (2 µg/ml) (BF), or monensin (2 µM) (MN). Levels of palmitoylated c-Met are demonstrated by Western blotting.

Inhibition of palmitoylation leads to the post-translational downregulation of c-Met seemingly through a block in intra- or post-Golgi transport.

In order to determine how inhibition of palmitoylation affects c-Met, we treated DU145 prostate cancer cells with 2BP over time. Western blot analysis revealed 2BP treatment leads to a progressive loss of total c-Met protein (Fig. 2A). We next wanted to analyze multiple levels of expression, including transcription, translation, as well as internalization and degradation, to determine how inhibition of palmitoylation downregulated c-Met. Using real-time quantitative PCR we have shown that transcription rates of c-Met are not influenced by 2BP treatment (data not shown). A Click-chemistry-based technique for detecting newly synthesized protein was used to determine that the rate of protein synthesis remains constant in the presence of 2BP (Fig. 2B). Taken together, these data indicate the downregulation must occur post-translationally. Consistent with this, confocal microscopy (Fig. 2C) and surface biotinylation experiments (data not shown) show that newly synthesized c-Met accumulates in the Golgi and is lost from the plasma membrane at a basal rate. Additional experiments suggest a role for ectodomain shedding in the loss of c-Met from the plasma membrane (data not shown). We hypothesize that preventing c-Met palmitoylation causes a build-up in the Golgi that is ultimately targeted for degradation. Future studies will address the mechanism of degradation.

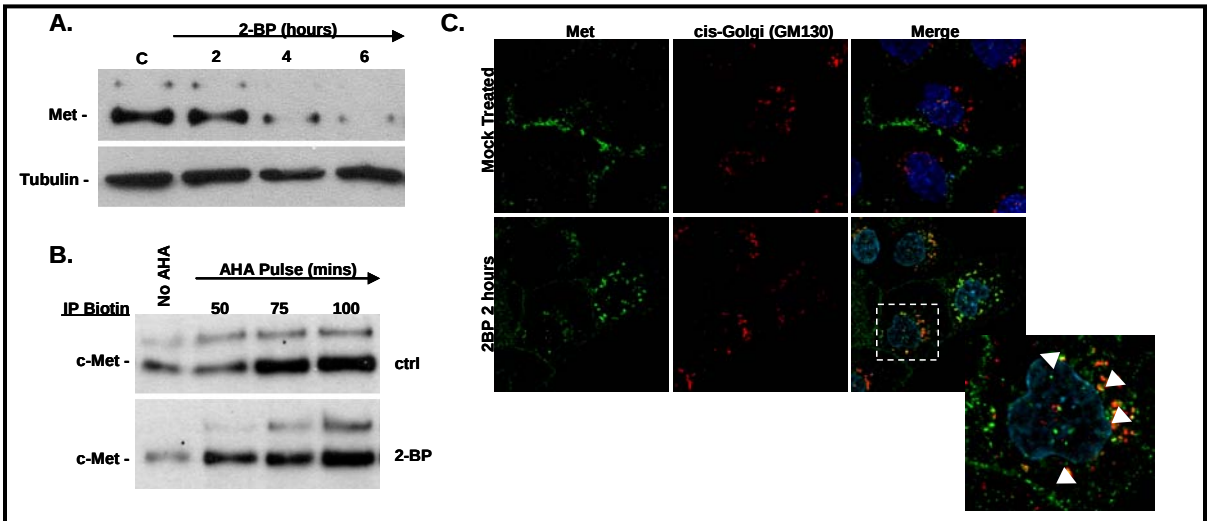


Figure 2. Inhibition of palmitoylation lowers total c-Met protein levels post-translationally and leads to accumulation of c-Met in the Golgi. A.) DU145 prostate cancer cells were treated with 100 µM 2BP for 2, 4, or 6 hours and c-Met expression was analyzed by Western blotting. B.) A Click-chemistry technique was used to label (AHA) and immunoprecipitate newly synthesized protein from DU145 cells in the presence or absence of 100 µM 2BP. The rate of c-Met synthesis is indcated by Western blot analysis. C.) DU145 cells were incubated with or without 100 µM 2BP for 2 hours and then fixed. c-Met (green) distribution was analyzed by costaining with the cis-Golgi marker GM130 (red). DAPI Representative 60x confocal immunofluorescence microscopy images are shown and sites of colocalization are highlighted by arrowheads.

Continuing Work

Since the last annual report submission the primary objective has been the design of palmitoylation defective c-Met mutants by site-directed mutagenesis of cysteine residues. These mutants have been made and are currently being tested for effects on c-Met palmitoylation status. Identification of a palmitoylation defective c-Met mutant will allow for a more accurate description of the influence palmitoylation has on c-Met trafficking, stability, and ultimately oncogenic potential. Palmitoylation defective mutants will be used in animal models of c-Met driven tumor growth (Aim 2). In addition, future work toward identifying the enzyme responsible for palmitoylation of c-Met will provide a new specific target for downregulating this receptor.

Also as part of Aim 2, I have optimized antibody titers for immunohistochemistry analysis on commercially-acquired (US BioMax) prostate cancer tissue arrays incorporating all stages of disease. We are currently acquiring data to be scored by our collaborating pathologist (CV included with initial proposal). Knowing that c-Met and FASN expression are frequently elevated in prostate cancer independently, it is important to test if their expression patterns overlap at the cellular level. Identifying a correlation between c-Met and FASN expression in vivo will support the significance of their connection in vitro. An established correlation will also provide foundation for future work into linking de novo fatty acid synthesis with c-Met palmitoylation. One could theorize that FASN could be used as a biomarker for more stabile and active c-Met, and therefore potentially more metastatic prostate cancer. More importantly, demonstrating in vivo that FASN-derived palmitate is preferentially utilized for c-Met acylation in cancer would highlight a novel modality for targeting c-Met expression.

Predoctoral Training Progress

-At the start of this funding year all predoctoral coursework had been completed as indicated in the initial application. I have no new coursework to declare.
-Current training is focused on mentorship, grantsmanship, presentation skills, and laboratory technique.

- -Present and participate in weekly cancer research journal club.
- -Present and participate in weekly departmental seminar series.
- -Continued training by my advisor during weekly research meetings.
- -Mentor students and technicians in the laboratory on a daily basis.
- -Participate in grant writing workshops provided by the LSUHSC-S graduate school.
- -Obtained training certification for the proper handling and experimental use of animals.
- -Participated in science advocacy Hill Day event in Washington, D.C. sponsored by American Society for Biochemistry and Molecular Biology (ASBMB).
- -Written and reviewed numerous grant proposals, review papers, book chapters, and primary research articles.

Key Research Accomplishments

-Determined c-Met to be a palmitoylated protein.
-Determined how blocking c-Met palmitoylation effects the protein's expression.

-Engineered twelve c-Met mutant constructs to identify palmitoylated residue/s, and to definitively demonstrate the functional outcome of preventing c-Met palmitoylation

Reportable Outcomes

Through the support of this predoctoral fellowship, I can claim the following outcomes:

Coleman, D.T., and Cardelli, J. c-Met Protein Expression is Regulated by Palmitoylation in Prostate Cancer Cells. Proceedings of the American Association of Cancer Research, Abstract 7446, April 2012. (Appendix i)

Coleman, D.T., and Cardelli, J.A. Regulation of c-Met by fatty acid synthase activity and palmitoylation. Proceedings of the American Association of Cancer Research Tumor Invasion and Metastasis Special Conference. Abstract 247210_1, January 2013. (Appendix ii)

Coleman, D.T., Gray, A.L., and Cardelli, J.A. Palmitoylation Regulates the Trafficking and Stability of the Receptor Tyrosine Kinase c-Met. Research article in preparation. (Appendix iii, Chapter 2.)

Successfully wrote and defended PhD thesis as the final requirement for PhD degree. Coleman, D.T. The c-Met receptor tyrosine kinase is regulated by fatty acid synthesis and palmitoylation. A Dissertation. Commencement ceremony to be held May 25th 2013. (Appendix iii)

Jason A. Cardelli Award for Excellence in Cancer Research. Feist-Weiller Cancer Center, Shreveport, LA. 2013.

Departmental nomination for Chancellor's Award (highest graduate student honor at LSU). Recipient to be announced at commencement, May 25th 2013.

Conclusions

Over the last several years, increasingly compelling evidence has been published showing that the c-Met receptor is an important therapeutic target for prostate cancer. The data acquired throughout the last reporting year have identified inhibition of palmitoylation, directly, or through inhibition of FASN activity, as a potential therapeutic strategy for downregulating the Overexpression of c-Met often observed in prostate cancer and especially prostate cancer bone metastases. From the data acquired over the last year, our laboratory's model has c-Met being palmitoylated as a precursor in the ER and this fatty acid attachment is required for its stability and proper trafficking out of the Golgi toward the plasma membrane. Blocking palmitoylation causes an accumulation in the Golgi and eventual degradation through a yet to be determined pathway. As far as we are aware, this is the first evidence for the palmitoylation of an oncogenic receptor tyrosine kinase. Future work will be aimed at 1.) identifying the specific cysteine residue/s palmitoylated, 2.) better defining the intracellular site and kinetics of c-Met palmitoylation and

degradation with higher resolution, and 3.) establishing proof of principle data in animals models using wt or palmitoylation-defective mutants of c-Met as laid out in the proposal. Ultimately, the support of this predoctoral fellowship has led to my fulfilling the requirements of a PhD degree for which I am immensely grateful. I will be transitioning into the position of an academic postdoctoral fellow having acquired superb training.

Appendix ii

AACR Tumor Invasion and Metastasis Special Conference

c-Met Requires Palmitoylation for Proper Stability and Trafficking in Cancer Cells

Department of Microbiology and Immunology and The Feist-Weiller Cancer Center
Louisiana State University-Health, Shreveport, LA
David T. Coleman and James A. Cardelli

The influence of growth factor receptors on cancer progression as both early promoters as well as drivers of late-stage invasion and metastasis has been thoroughly studied over the last several decades. Overexpression and activating mutations of several receptor tyrosine kinases (RTKs) are commonly detected in most cancer types. Despite this, there still remains a great deal not fully understood about RTK expression and regulation that may reveal novel strategies for therapeutic targeting. In particular, c-Met is an RTK whose downstream signal transduction can promote both mitogenic and motogenic phenotypes in cancer cells and whose expression is correlated with poor prognosis and resistance to therapy. c-Met is activated by either autocrine or paracrine ligand stimulation. Alternatively, receptor overexpression allows for ligand-independent dimerization and therefore constitutive activation.

In addition, multiple reports have identified the enzyme fatty acid synthase (FASN) as being commonly overexpressed in prostate cancer, and that this aberrant expression is an early event that becomes more pronounced with aggressive androgen-independent and metastatic disease. FASN is the sole enzyme responsible for *de novo* synthesis of the 16-carbon saturated fatty acid palmitate. In cancer, de novo lipids are more selectively partitioned into lipid rafts as phospholipids as well as utilized for post-translational acyl-modifications of signaling proteins. Previous findings have led us to identify a novel mechanism by which FASN activity regulates c-Met expression. Our work has determined that inhibition or shRNA knockdown of FASN results in a post-translational downregulation of already synthesized c-Met protein. This downregulation is prevented by the addition of exogenous palmitate.

Based on these findings we have subsequently acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation regulates its stability. Inhibition of palmitoylation reduces the expression of c-Met in multiple cancer cell lines. This protein loss occurs post-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies, confocal microscopy, and metabolic-ortholog labeling we determined that inhibition of palmitoylation reduces the stability of newly synthesized, c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol suggest c-Met itself is palmitoylated. Observing palmitoylation kinetics has provided evidence that c-Met is palmitoylated in the ER prior to cleavage of the 170kd c-Met precursor into its mature 140kd form. Taken together, these findings suggest inhibition of palmitoylation or FASN activity could be a novel target for preventing invasion and metastasis driven by c-Met overexpression.

The c-MET Receptor Tyrosine Kinase is Regulated by Fatty Acid Synthesis and Palmitoylation

A Dissertation

Submitted to the Graduate Faculty of the Health Sciences Center of Louisiana State University and Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology and Immunology

by
David Thomas Coleman
B.S., Mississippi State University, 2006
May, 2013

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abstract

The influence of growth factor receptors on cancer progression as both early promoters as well as drivers of late-stage invasion and metastasis has been thoroughly studied over the last several decades. Overexpression and activating mutations of several receptor tyrosine kinases (RTKs) are commonly detected in most cancer types. Despite this, there still remains a great deal not fully understood about RTK expression and regulation that may reveal novel strategies for therapeutic targeting. In particular, c-Met is an RTK whose downstream signal transduction can promote both mitogenic and motogenic phenotypes in cancer cells and whose expression is correlated with poor prognosis and resistance to therapy. c-Met is activated by either autocrine or paracrine ligand stimulation. Alternatively, receptor overexpression allows for ligand-independent dimerization and therefore constitutive activation.

In addition, multiple reports have identified the enzyme fatty acid synthase (FASN) as being commonly overexpressed in prostate cancer, and that this aberrant expression is an early event that becomes more pronounced with aggressive androgen-independent and metastatic disease. FASN is the sole enzyme responsible for *de novo* synthesis of the 16-carbon saturated fatty acid palmitate. In cancer, de novo lipids are more selectively partitioned into lipid rafts as phospholipids as well as utilized for post-translational acyl-modifications of signaling proteins. Previous findings have led us to identify a novel mechanism by which FASN activity regulates c-Met expression. Our work has determined that inhibition or shRNA knockdown of FASN results in a post-

translational downregulation of already synthesized c-Met protein. This downregulation is prevented by the addition of exogenous palmitate.

Based on these findings we have subsequently acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation regulates its stability. Inhibition of palmitoylation reduces the expression of c-Met in multiple cancer cell lines. This protein loss occurs post-transcriptionally and is associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies, confocal microscopy, and metabolic-ortholog labeling we determined that inhibition of palmitoylation reduces the stability of newly synthesized, c-Met as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol suggest c-Met is palmitoylated. Observing palmitoylation kinetics has provided evidence that c-Met is palmitoylated in the ER prior to cleavage of the 170 kd c-Met precursor into its mature 140 kd form. Taken together, these findings suggest inhibition of palmitoylation or FASN activity could be a novel target for preventing invasion and metastasis driven by c-Met overexpression.

Literature Review

Review of c-Met: Biosynthesis, Regulation, and Physiology

Characterization of the c-Met Oncogene

c-Met was first identified in 1984 as a proto-oncogene in the laboratory of George Vande Woude. The protein was detected as an oncogenic fusion protein (TPR/Met) resulting from a chemical carcinogen-induced chromosomal translocation [1]. The translocated promoter region (TPR) fusion with c-Met forms a constitutively active c-Met dimer. Isolation of the translocated cDNA product led to the identification of the native-form full-length receptor [2, 3]. Subsequently, much effort was given to understanding the biosynthesis and processing of this important protein. The *MET* proto-oncogene is located on chromosome 7 (7q21-q31). Work by Comoglio et al. determined that c-Met was synthesized as a 170 kd precursor protein. This precursor protein becomes extensively glycosylated within the endoplasmic reticulum (ER) before being transported into the cis-Golgi cisternae. Within the late cisternae (medial- or trans-) of the Golgi the 170 kd precursor protein becomes proteolytically processed by furin into a 50k d α-chain and a 140 kd β-chain which are linked by disulfide bonds to form the mature 190 kd functional protein [4-6]. The full domain structure and functional map for c-Met protein has been reported (refer to Fig. 1) [7]. The mature form continues through the Golgi into secretory vesicles which deposit the single transmembrane spanning receptor tyrosine kinase at the plasma membrane (Fig 3.). There is some evidence that the c-Met receptor resides within cholesterol-rich lipid raft membrane domains [8-10]. The mature 190 kd protein was found to be autophosphorylated at tyrosine residues within intracellular

domains and phosphorylated by other kinases at serine residues [11-13]. The cognate ligand for c-Met was determined to be a secreted homodimeric glycoprotein known as both scatter factor and hepatocyte growth factor (HGF) due to its ability to promote motility and cell morphology changes [14-17]. The binding affinity of HGF was later determined to require extracellular proteolytic cleavage by urokinase [18]. HGF is principally secreted by stromal fibroblasts to regulate normal tissue development and wound repair in conjunction with its target receptor c-Met [16, 19]. It had been observed that c-Met was often overexpressed in spontaneous transformants of 3T3 cells and ectopic expression of c-Met cDNA was subsequently shown to transform 3T3 cells. Although, forced expression of c-Met increased tumorigenicity of non-tumorigenic cell lines in nude mice, coexpression of both c-Met and HGF together produced a much more robust tumorigenic phenotype [20].

c-Met Signaling

Dimerization of the c-Met receptor catalyzes a series of events that culminate in motogenic and mitogenic phenotype changes (Fig 2.) [8, 21, 22]. In greater detail, dimerization of individual c-Met molecules, normally facilitated by ligand binding, lowers the activation energy necessary for autophosphorylation of sets of tyrosine residues at positions 1234/1235 as well as 1349/1356, the latter of which serve as docking sites for recruited downstream signaling components. Adaptor proteins Gab1, Grb2, Src, Shp2, and others dock at these c-terminal phosphorylated residues to facilitate activation of kinase cascades terminating with gene expression changes or dead-end protein activation. c-Met activates the Ras-Raf-MEK-ERK cascade which terminates with ERK translocation into the nucleus where it stabilizes a number of transcription

factors through phosphorylation [23]. In general, the targeted transcription factors initiate G1-S cell cycle progression [24]. The JNK and p38 MAPKs are also activated in response to c-Met phosphorylation and contribute to numerous cellular processes. Phosphoinositide 3-kinase (PI3K) is recruited to the plasma membrane in response to phosphorylated c-Met where it acts as a lipid kinase to relocate pleckstrin homology domain (PH domain)-containing proteins to the plasma membrane for spatially regulated activity. In particular, the PH domain-containing protein and Ser/Thr kinase Akt is recruited and activated by PI3K activity propagating the cascade to anti-apoptotic effector proteins, cell cycle regulators, as well as mediators of protein synthesis and metabolism such as mammalian target of rapamycin (mTOR) [25-27]. The MAPK and PI3K pathways stimulate actin polymerization and branching, required for cell motility, through CDC42 and Rac activities [28]. Additionally, direct phosphorylation of focal adhesion kinase (FAK) by c-Met is required for HGF-driven motility and invasion [29, 30]. Numerous reports indicate Src-family kinases including Fyn function downstream of c-Met activity, phosphorylating FAK and paxillin to alter cell shape and enhance cell motility [31]. STATs directly associate with dimerized c-Met which tyrosine phosphorylates the transcription factors initiating STAT homodimerization and nuclear translocation. Numerous genes responsible for cell differentiation and proliferation are sensitive to STAT nuclear activity [32, 33].

Physiological Role of c-Met: Development and Wound Repair

The cumulative effects of c-Met signaling are required for normal physiology controlling a number of related processes. During embryogenesis, cells must migrate and morph to shape the developing organs. c-Met-null mouse embryos have been shown to

die *in utero* due to improper liver development and dysregulated trophoblast arrangement, highlighting the importance of this protein in the development of proper embryonic architecture. In adults, the tissue remodeling and cell migration required to reform normal tissue architecture during wound repair, is somewhat analogous to the events of embryonic development. In addition to enhanced motility, tissue development and repair require the ability of cells to survive anoikis, or apoptotic cell death in response to lost adhesion, as they detach from neighboring cells and extracellular matrix (ECM) components. During this invasive growth, it is necessary for cells to proteolytically remodel the surrounding restrictive ECM. Findings from our laboratory have shown that c-Met signaling can redistribute protease-rich lysosomes to the plasma membrane where they fuse and release enzymes to digest surrounding matrix proteins [34, 35]. Once resettled, cells must repopulate the tissue site, requiring robust mitotic cycling manifested by c-Met activity [24]. Similarly, epithelial tubulogenesis, which is the formation of hollow tubular structures necessary for angiogenesis; mammary gland formation; as well as kidney, pancreas, and liver regeneration is controlled, to great extent, by HGF/c-Met signaling. In fact, in vitro; migration, invasion, and tubulogenesis of human umbilical vein endothelial cells (HUVECs) are prevented by treatment with c-Met blocking peptides [36]. Tubulogenesis requires cells to take on a polarized morphology, invade surrounding ECM, and interact with other cells to form a continuous luminal structure [22]. The transition of unpolarized cells to a polarized and invasive phenotype exhibits characteristics of a postulated phenomenon called an epithelial to mesenchymal transition (EMT) often cited in cancer invasion [37]. Epitomizing the potency of this protein, it has been shown that c-Met is essential for hepatocyte

repopulation and tissue remodeling in partial liver transplant donors, a process that can regenerate 80% of the removed tissue in less than 1 week [38]. The physiological processes discussed herein, normally occur in response to extracellular cues from cells of the regional stroma; such as HGF secretion from fibroblasts, that, as discussed, activates c-Met, propagating the kinase cascades that modulate activity and expression of proteins involved the aforementioned processes [24, 39].

Regulation of c-Met Expression

The potency of c-Met function has led to the evolutionary development of many strategies to maintain its accurate expression and activity. These strategies include proteolysis, transcriptional and translational regulation, enzymatic dephosphorylation, and internalization. Several of these measures will be discussed here in more detail.

The synthesis of c-Met is transcriptionally regulated downstream of growth factor signaling and environmental conditions. The transcription factors SAP1, Pax3, Ets1, p53, Smad, and Sp1 have all been identified as positive regulators acting at the c-Met promoter [40, 41]. Hypoxia-inducible factor-1 (HIF-1) acts in response to low oxygen tension to upregulate c-Met expression [42, 43]. Conversely, interferon signaling and oxidative stress can repress c-Met transcription by upregulating the nuclear protein, and histone deacetylase recruiter, Daxx, which targets the c-Met promoter, thereby demonstrating a role for epigenetics in c-Met expression [44].

There is evidence that the complex 5' untranslated region (UTR) of c-Met mRNA allows for a level of cap-dependent translational control, under certain context. This was exhibited by knockdown of elongation initiation factor 4E (eIF4E) restricting c-Met synthesis [45]. Our laboratory has recently found that the monocarboxylate transporter 1

(MCT1) is required, post-transcriptionally, to maintain expression of c-Met in prostate cancer cells (unpublished data). Post-translationally, reports from the Vande Woude laboratory, among others, have demonstrated that c-Met requires association with certain chaperone proteins such as heat shock protein 90 (Hsp90) for maintained stability and plasma membrane expression. Treatment with geldanamycins to inhibit Hsp90 results in a greatly reduced c-Met half-life [46]. Moreover, c-Met activity can be regulated by phosphatases to shutdown signaling output. Phosphorylation of S985 within the c-Met juxtamembrane domain by protein kinase C (PKC) results in dephosphorylation of the tyrosine residues responsible for downstream signaling [47]. protein phosphatase 2A (PP2A) has been shown to dephosphorylate Ser985, thereby promoting an activated c-Met [48, 49].

During the autophosphorylation cascade that occurs in response to dimerization, the juxtamembrane Y1003 becomes phosphorylated increasing the receptor's affinity for the E3-ubiquitin ligase c-Cbl [50-52]. Monoubiqutination of the receptor initiates a series of events that destines the receptor for degradation. At the same time, ubiuquitin-interacting domain-containing proteins, most importantly HGF-regulated tyrosine kinase substrate (Hrs), associated with ubiquitinated forms of the receptor to hold them within these internalization vesicles and promote degradation versus recycling [53-55]. In fact, activation of c-Met leads to phosphorylation of Hrs which is required for internalization [56]. c-Cbl-dependent internalization typically occurs through a dynamin-dependent scission of clathrin-coated pits. Although this internalization scheme seems to be the default mechanism for c-Met internalization, there are a multitude of other internalization strategies possible under unique conditions. For instance, receptors can be internalized in

bulk by macropinocytosis or membrane ruffling, and selective lipid-raft associated receptors can be internalized into caveolae [57].

In concert with internalization, the molecular mechanisms controlling the route of degradation or; alternatively, recruitment to recycling vesicles, is equally convoluted (refer to Fig. 3. and reviewed [58]). Given the importance of internalization and degradation on regulating the levels and activity the c-Met receptor, much effort has gone into understand this process. The research groups of Peter Parker, Morag Park, and Michael Clague have led this area and detailed models of this process have been outlined [58, 59]. Upon activation of the receptor, phosphorylation at Y1356 recruits the adaptor protein Grb2. The proline-rich region of the ubiquitin ligase c-Cbl is attracted to the SH3 domain of Grb2 where it is phosphorylated initiating a conformational change of the ubiquitin ligase [60]. c-Cbl is then able to associate with a phosphorylated juxtamembrane tyrosine residue (Y1003) on c-Met. This phosphotyrosine residue is essential for receptor degradation, and the initial recruitment of Grb2 is required for endocytosis. In addition to ubiquitinating the receptor, c-Cbl association serves to nucleate a complex of clathrin-regulated endophilins and CIN85 to concentrate c-Met into clathrin-coated pits. The endophilins function to curve and invaginate the pits to allow scission and endocytosis of the receptor-containing vesicle. Ubiquitination of c-Met is a rapid event, easily detectable within 10 minutes of HGF treatment, whereas significant degradation can take 1 to 4 hours [53, 54, 56]. Binding of phosphorylated Hrs to ubiquitinated c-Met promotes fusion of the early endosome vesicle with multivesicular endosomes through an interaction with the endosomal sorting complex required for transport (ESCRT)-1. This is a common event for degradation of many different

ubiquitinated plasma membrane proteins [55]. Once internalized, activated receptors are either recycled back to the plasma membrane or degraded by the cytoplasmic 26S proteasome or in proteolytic lysosomes. In the case of c-Met, the precise mechanism of degradation has been nebulous for many years. Using MG132 and lactacystin, inhibitors to the 26S proteasome complex, researchers suggested c-Met degradation occurred solely through this route; however, more recent studies suggest the proteasome only plays an indirect role in degradation [61, 62]. Research by Hammond et al. has revealed that lactacystin treatment prevents degradation by promoting the recycling of c-Met from early endosomes back to the plasma membrane, and, in fact, lysosomal proteolysis is responsible for direct c-Met degradation [56, 63-65]. The confusion arises in that lactacystin treatment was preventing c-Met degradation only because it led to the reduction of Hrs within endosomes causing a bias toward the recycling of c-Met back to the plasma membrane. However, Kermogant et al. followed these findings with data, in the same cell line, suggesting that c-Met was directly degraded entirely by the proteasome and proteasomal activity did not affect the internalization nor sorting of c-Met [61]. This research group also found that PKC activity and microtubules were required for the trans-cytosolic trafficking of c-Met required for degradation. What seems to be most important from these studies is that there is clearly potential for c-Met degradation to occur equally well through lysosomal or proteasomal pathways given context dependence and possible compensatory activity. It is also important to note, that these studies on c-Met degradation have illustrated a level of uniqueness to the post-translational regulation of c-Met expression over other receptor tyrosine kinases (RTKs), principally the ErbB family of RTKs [66-69].

The c-Met receptor is also sensitive to proteolytic cleavage at the plasma membrane as a mechanism to both downregulate the signal as well as yield bioactive protein fragments with proposed independent functions. It has been shown that caspases can cleave c-Met, separating the intra- and extra- cellular domains forming a proapoptotic fragment termed p40 c-Met [70, 71]. Additionally, metalloproteinases, specifically ADAM17 of the 'a disintegrin and metalloproteinase' family, have been shown to cause ectodomain shedding of the receptor. Initially thought to be facilitated by the tissue inhibitor of metalloproteinases (TIMP)-3, it has more recently been found that a disintegrin and metalloproteinase (ADAM) subfamily members, specifically ADAM17 and ADAM10, are able to cleave c-Met from the cell surface. This ectodomain release can be initiated through MAPK activity in response to EGF treatment, integrin ligation, *Helicobacter pylori* infection as well as partially induced by stimulation with HGF or a known c-Met-activating monoclonal antibody [72-74] (300, 301, 303). There is additional evidence that the soluble ectodomain can act as a decoy fragment that competes for ligand; therefore, it has a negative effect on the c-Met signaling axis [75]. Subsequent to ADAM17 cleavage of the extracellular domain, presenilin-dependent intramembrane proteolytic activity of the γ-secretase complex frees a 50 kd intracellular fragment to be degraded by the proteasome or lysosome [76, 77]. The significance of this mechanism for downregulation is highlighted by studies demonstrating reduced transformative potential of c-Met when artificial means of shedding are induced [73]. Moreover, shed fragments of c-Met can be isolated from urine and plasma, and their presence correlates with malignant potential in mice harboring human tumor xenografts [78].

Incidence and Contributions of c-Met in Cancer Progression

Suggested by the characterization of cancer as a wound that does not heal, when unconstrained, the c-Met signaling axis can cause and/or exacerbate the development of cancer and its spread throughout the body [24]. As previously mentioned, the downstream outcomes of c-Met activity promote cell proliferation, resistance to anoikis, and EMT-associated invasive growth - all characteristic of cancer. In fact, despite the critical role for c-Met in healthy physiology, the vast majority of research publications are related to its clinical significance in cancer. There is an abundance of data implicating c-Met in tumor initiation and invasive progression. Numerous cell lines have been shown to become tumorigenic and metastatic in mouse models when ectopically overexpressing c-Met. Conversely, pharmacological or genetic downregulation of c-Met reduces the tumorigenic potential of certain cancer cell lines [79]. Incidents of most epithelial cancers, including ovarian, hepatic, pancreatic, lung, rectal, gastric, prostate, breast, and colorectal as well as certain hematopoietic cancers, including B-cell lymphoma, acute myeloid leukemia, and multiple myeloma, have been associated with dysregulated c-Met [80]. Expression of the receptor frequently correlates directly with disease progression and inversely with patient outcome [79-83]. The receptor becomes abnormally expressed and/or activated by failure of one or more of the regulation mechanisms discussed above. Germline or somatic mutations of the *MET* gene as well as chromosomal rearrangements can result in constitutive phosphorylation or dimerization [2, 3, 84-86]. Transcriptional upregulation and *MET* gene copy number amplification can cause excessive expression of the receptor which, in and of itself, can result in ligand-independent dimerization and activation [87-89]. Similarly, ineffective downregulation

of activated receptor can result in the accumulation; either through defective trafficking mediators or mutations in the c-Met juxtamembrane domain required for internalization and degradation [51, 90-93]. Excessive ligand secretion; either in a paracrine fashion from fibroblasts, or through aberrant HGF expression from the tumor cells, can also hyperactivate the c-Met signaling axis [94, 95].

The oncogenic manifestations of dysregulated c-Met are extensive, as can be assumed from its pleiotropic nature discussed above [96-98]. The ultimate result, though, is an EMT that is molecularly comparable to cellular dedifferentiation into a stem-cell like phenotype [99]. In recent years, there has been a great deal of attention toward the ability of stem-like cancer cell populations to resist therapeutic strategies as dormant microlesions, to repopulate frank tumors during treatment relapse, and to more efficiently seed metastatic tumors [100-103]. These three attributes represent fundamental hurdles limiting success in clinical oncology at the current time, thereby emphasizing the importance of targeting c-Met in cancer treatment. The association of c-Met with resistance to different therapies, particularly targeted therapies, has become an increasingly common theme in reports from cell culture and clinical-trial follow-up reports. In particular, c-Met overexpression has been shown to circumvent biological or pharmacological blockade of ErbB family member (EGFR, Her2, and Her3) phosphorylation by acting as an atypical heterodimerization partner [104-110]. In addition, amplified c-Met is a clinically observed mechanism of resistance to BRAF inhibitors in aggressive melanoma [111, 112]. Most clinical observations and in vitro models indicate that overabundance of the receptor alone is able to confer therapeutic resistance, independent of mutant activity. This points to potential benefit in shifting

therapeutic strategies from blocking activity to restricting expression. There are currently 10 specific c-Met inhibitors in different stages of clinical trial, with several showing great promise even controlling advanced metastatic disease [80, 113]. Similar success has been reported with several of the 11 non-specific c-Met tyrosine kinase inhibitors currently being evaluated in clinical trials. Particularly those with affinity for the vascular endothelial growth factor receptor (VEGFR) in addition to c-Met, such as cabozantinib, have exhibited significant improvement over standard treatment. Progression free survival improved from 6 weeks to 21 weeks with cabozantinib treatment in castration-resistant prostate cancer patients (P=.0007) [80]. Despite exciting successes with new c-Met targeting therapeutics, it has become clear over the last decade that single-modality treatment strategies, targeting kinase activity, will not be adequate to control the majority of cancers the majority of the time. This understanding supports the need for continued progressive research into the molecular life of this, and other, therapeutic protein-targets.

Review of Fatty Acid Synthase: The Lipogenic Phenotype in Normal and Cancer Physiology

Fatty Acid Synthase: The Enzyme

The FASN gene product forms a homodimer of which each monomer acts as a multifunctional protein containing 7 catalytic sites for stepwise condensation of acetyl CoA and malonyl CoA subunits into long chain fatty acids (Fig 4.). The bulk product of FASN activity is the 16-carbon saturated fatty acid palmitate, but a small fraction of the fatty acids synthesized are the 14- or 18-carbon saturated fatty acids myristate or stearate respectively. The lipogenic phenotype is a metabolic state characterized by elevated fatty acid and sterol synthesis. Both pathways utilize acetyl CoA as the primary carbon source and are; therefore, coordinated with the glycolytic pathway [114]. Lipogenesis functions both as a means of storing energy, as well as a source of hydrophobic protein modifiers and membrane building blocks. More specifically, *de novo* synthesized fatty acids can be used for membrane biogenesis, protein acylation, synthesis of lipid signaling mediators, maintenance of the cellular redox state, and storage of excess energy. The lipogenic phenotype is regulated by a master transcription factor, sterol-regulatory element binding protein (SREBP)-1. This transcriptional factor regulates expression of most of the enzymes responsible for fatty acid and cholesterol synthesis including ATP citrate lyase (ACLY) and acetyl-CoA carboxylase (ACC). ACLY synthesizes acetyl-CoA from mitochondrial citrate to be a carbon substrate for FASN. A subset of this acetyl-CoA is converted to the 2-carbon substrate malonyl-CoA by ACC in the rate-limiting regulatory step of fatty acid synthesis. Under particular metabolic conditions, fatty acids, often those stored linked to glycerol in the form of neutral triglycerides, must be oxidized in

order to free their bound energy for use in other cellular tasks. This process, known as β-oxidation, occurs in the mitochondria. Acylglycerols are hydrolyzed to free fatty acids which are translocated into the mitochondria by the tightly regulated enzyme carnitine o-palmitoyltransferase (CPT)-1. In a healthy adult consuming an adequate diet, FASN is expressed at low to undetectable levels in most tissues. FASN is also essential for fetal lung surfactant production, fat enrichment of breast milk, the conversion and storage of dietary energy principally in liver and adipose tissue, and its expression can fluctuate in highly proliferative cells such as those of the colonic epithelium [115].

Fatty Acids

There are several physiologically relevant fatty acids in humans (refer to Table 1. for a structural comparison). The three main long-chain saturated fatty acids include myristate, palmitate, and stearate. Palmitate and stearate can be elongated in human cells to very long chain fatty acids composing ceramide, glycolipids, and sphingolipids which are common components of membranes and signaling molecules. Much of the research on fatty acids in normal and disease physiology is plagued by inaccuracies with respect to the concentrations and ratios of different fatty acids used. Physiological concentrations of fatty acids in serum range from 50-750 μM complexed to albumin. Only roughly 30 percent of all circulating fatty acids are long-chain saturated fatty acids, whereas the rest are essential dietary fatty acids with varying degrees of saturation [116]. Numerous reports detail the effects specific fatty acid species or slightly distinct ratios of fatty acid combinations can have on cellular physiology. This emphasizes the tremendous ability cellular components have discerning subtle differences in chain length and degree of saturation. For example, in general, the Ω-9 monounsaturated 18-carbon fatty acid oleate

has a mitogenic effect and promotes PI3K activity, whereas the 16-carbon saturated fatty acid tends to halt proliferation and lower PI3K activity. The major fault with these studies is maintaining physiologically relevant concentrations and environmental context. Study into the biological effects of fatty acids must make every effort to experiment with accurate concentrations and realistic ratios of fatty acid species. Furthermore, it is clear that cellular biology of healthy cells can be very different from that of cells in a diseased state. Oleate and polyunsaturated fatty acids (PUFAs), rich in a Mediterranean diet, can reduce FASN activity. This activity has been shown to reduce Her2 expression as a potential explanation for lower rates of breast cancer in the Mediterranean; however it is doubtful whether the supraphysiological exposure of cell culture to individual fatty acid species can be extrapolated to possible effects of dietary fatty acids. [117, 118].

Influence of *de novo* Fatty Acid Synthesis in Normal Physiology

When considering the functions of FASN broadly, it becomes clear that this enzyme can influence most, if not all, cellular functions. Changes in FASN expression and activity can fluctuate the availability of lipid signaling molecules such as phosphatidylinositol (3,4,5)-triphosphate (PIP_3), lysophosphatidic acid (LPA), and prostaglandins. These molecules are involved in major signaling pathways that have roles in everything from cell growth and survival to cell motility and smooth muscle cell constriction. Through anaplerotic reactions and energy storage, FASN can regulate cellular redox state and ATP availability. There are several pieces of evidence, which will be discussed more in-depth in this chapter, that FASN activity can actually promote protein acylation which is known to spatially regulate numerous signaling pathways.

Given the immense responsibility of regulated lipogenesis, it is not difficult to see how cellular abnormalities could occur when dysregulated.

A pivotal study adding to the understanding of *de novo* fatty acid synthesis in development was accomplished by Salih Wakil's group. The research team was able to generate FASN knockout mice and analyze the different developmental patterns of homo- and heterozygotes. Heterozygotes were born at a less than expected Mendelian rate indicating that having only one functional allele confers an impediment during development. However, the $FASN^{-/+}$ that were birthed had a 50% reduction in FASN expression and a similar reduction in cellular lipogenic activity as determined by ^{14}C-acetate incorporation. The 50% reduction in FASN levels indicates this protein is affected by gene dosage. All homozygous FASN knockout mice were aborted at an early stage of development, typically prior to implantation, indicating *de novo* lipogenesis and/or FASN expression is essential for embryonic development. Interestingly, feeding a diet rich in saturated fatty acids did not prevent the embryonic lethality of the hetero- or homozygotes demonstrating a unique role for *de novo* fatty acid synthesis compared to exogenous dietary sources. Wildtype embryos were also analyzed for distribution of FASN expression. At different stages of embryonic development, immunohistochemistry analysis of FASN expression indicated that the protein was elevated consistently in regions of tissue outgrowth, morphogenesis, and remodeling [119]. These processes are known to require signaling through a number of growth factor receptors including those for Wnt ligands, fibroblast growth factor (FGF), bone morphogenic proteins (BMPs), and HGF among others. This single study calls attention to four major concepts, 1) fatty acids synthesized *de novo* are not functionally interchangeable with dietary sources of

fatty acid, 2) gene dosage can affect the levels of FASN, which will be explored further in the context of cancer-associated FASN, 3) in normal embryonic development, FASN is elevated in regions undergoing processes similar to those observed in wound-healing and cancer, and 4) that caution should be taken when considering drugs targeting FASN activity or expression due to possible teratogenic effects (4).

Identification of FASN as an Oncogene

It has been known for over a century that the conversion of normal cells to cancerous cells is accompanied by changes in metabolism . This was originally observed with respect to glycolysis, but much more recently, changes in lipogenesis as well as glutaminolysis have been included as major hallmarks of cancer cell reprogramming [115, 120]. Over 60 years ago it was found that most cancer cells produce 95% of their fatty acids *de novo*[121] . Granted, it was not until 1989 that FASN, first termed oncogenic antigen-519 (OA-519), was identified as a cancer-associated gene being frequently overexpressed in breast tumor cells [122, 123]. Since this time, elevated FASN has been detected in most cancer types. Many of the enzymes essential for the lipogenic phenotype are also abnormally expressed in cancer [124]. Herein, I will discuss the details of some of these findings, explain efforts made to exploit FASN as a therapeutic target, and try to explain possible mechanisms by which FASN becomes dysregulated and how cancer cells can possibly benefit from this.

FASN was suggested to be a strong candidate biomarker for prostate cancer based on whole tumor genome sequencing and gene copy number analysis comparing an array of cancer stages to benign prostate hyperplasia (BPH). There was a clear and consistent upregulation of FASN when comparing frank tumor samples to BPH [125]. Others have

demonstrated FASN expression tightly correlates with high Gleason score compared to low grade prostate cancer. Patient prostate samples including benign prostate hyperplasia, low grade prostatic intraepithelial neoplasia (PIN), high grade PIN, and invasive carcinoma were found to be positive in 0/87, 24/25, 26/26, and 82/87 respectively, and intensity correlated progressively with disease as well [126]. These data indicate FASN expression is an early event in prostate cancer that becomes progressively increased through the development of invasive and metastatic cancer. In agreement with this report, a follow-up study demonstrates that FASN mRNA levels are diagnostic for prostate cancer progression and, counter intuitively, this androgen-responsive enzyme is most highly expressed in castrate-resistant bone metastases [127]. Given that c-Met expression is exceptionally high in prostate cancer bone metastases and that c-Met inhibition exhibits potent efficacy against clinical bone metastases, it is reasonable to associate c-Met activity and FASN expression in the growth and survival of metastatic prostate cancer cells.

Roughly half of all prostate adenocarcinomas exhibit FASN gene copy gain, which is one explanation for the frequently observed elevated levels of FASN protein. Interestingly, although other enzymes, such as ACC and ACLY, involved in the regulation of fatty acid synthesis are closely associated on chromosome region 17q, only FASN gene amplification was commonly detected [128]. This would suggest that, although these enzymes are required for *de novo* fatty acid synthesis, greater changes in their expression isn't advantageous in the context of selection for cancer proliferation. Moreover, these enzymes may not be affected by gene dosage, but rather activity can be enhanced sufficiently without drastic expression changes.

Growth factor stimulation can also upregulate expression of SREBP-1 to promote lipogenesis [129]. Additionally, androgen stimulation uniquely upregulates FASN expression in prostate cancer cells by elevating Kruppel-like factor 5 (KLF5) expression. KLF5 directly interacts with SREBP-1 to enhance its activity specifically at the FASN promoter [130]. The 5-α reductase inhibitor dutasteride can be a highly effective antiandrogen therapy for prostate cancer. Interestingly, FASN, being an androgen-responsive gene, is one of the most commonly downregulated genes in response to dutasteride treatment and was shown to be a major cause of the dutasteride-induced cell death [131].

Hyperactivation of the PI3K/Akt pathway is a common occurrence in many cancer types including prostate cancer. Aberrant signaling can occur through deletion or inactivation of the tumor suppressor phosphatase and tensin homolog (PTEN), constitutive activation of growth factor receptors, or direct PI3K or Akt mutations. The PI3K pathway stimulates glucose uptake which will increase cellular ATP levels, inhibit AMPK activity, and bias fatty acid synthesis. In contrast, lower energy levels will activate AMPK and stimulate β-oxidation. Consistent with this, Watabe et al. have demonstrated that there is an inverse correlation between FASN expression and PTEN expression in prostate cancer tissue samples [132]. High levels of FASN in prostate cancer tissue samples was associated with elevated levels of nuclear phosphorylated Akt staining ($P<.001$) and these two correlated with prostate Gleason score ($P<.001$) [133]. In support of a functional role for FASN in driving prostate cancer cells, shRNA knockdown of FASN was shown to halt growth of LNCaP prostate cancer cells *in vitro* while having no effect on the proliferation of nonmalignant cultured skin

fibroblasts[134]. Similarly, ovarian cancer tissue samples FASN was found to be correlated with high phosphorylated AKT [135]. Several molecular studies have detailed a positive feedback regulation between PI3K activity and FASN expression and activity in both prostate and ovarian cancer cell lines. The PI3K pathway can promote the expression of FASN through the transcription factor SREBP-1 and enhance activity by increasing glucose uptake [135-137]. Demonstrating causation, the PTEN-null prostate cancer cell line LNCaP exhibits high levels of FASN, and ectopic reintroduction of PTEN transcriptionally lowers FASN levels [136].

FASN ectopic expression was shown to increase proliferation and anchorage-independent soft-agar growth of immortalized prostate epithelial cells (iPrEC) as well as prostate adenocarcinoma LNCaP cells. Moreover, when overexpressing FASN along with the androgen receptor (AR) the noncancerous iPrECs acquire tumorigenic potential and were shown to form invasive adenocarcinomas in 12 of 14 immunodeficient mice compared to 0 of 14 mice when only AR was expressed. Additionally, when FASN expression was driven by the prostate specific ARR2 probasin promoter, 44% of transgenic mice developed PIN beyond 7 months (P=.0028). FASN expression in the iPrECs conferred protection against apoptosis induced by chemotherapeutics as well as the intrinsic apoptosis pathway stimulators camptothecin and etoposide. In prostate cancer patient samples, FASN expression was inversely correlated with markers for apoptosis (P=.002) [138]. Together these data designate FASN as a bona fide oncogene in prostate cancer and an influence on chemotherapeutic resistance.

Targeting FASN for Cancer Therapy

Given the introduction to FASN in cancer, it is important to introduce the status of drugs designed to target this enzyme before further discussion (refer to Table 2. for a description of relevant compounds). There are a few inhibitors of FASN activity that have been discovered or synthesized, and each has limitations prohibiting widespread use as clinical therapeutics. One of these compounds is called C75. This compound is a mimetic of malonyl-CoA, a 2-carbon substrate of FASN. In addition to inhibiting FASN, C75 has been show to stimulate β-oxidation of fat stores through direct activation of CPT-1 in smooth muscle cells. Activation of CPT-1 in the hypothalamus mimics a satiated state which reduces the systemic release of neuropeptide Y. Together this works explains the observations that C75 causes severe anorexia and weight loss in animal models and it predicts the same problems if used with human patients [139-142]. Because of this, recent efforts by the Kuhajda laboratory have developed a C75 analogue, designated C93, which does not activate CPT-1 [143]. Studies with C93 have shown that it is able to inhibit growth of subcutaneous or orthotopic lung cancer xenografts without affecting weight gain as well as number and size of carcinogen-induced lung tumors in mice [143, 144]. *In vitro* cancer cell cytotoxicity was determined to be primarily due to accumulation of the FASN substrate malonyl CoA. Under an active lipogenic phenotype, ACC produces malonyl CoA from acetyl CoA units. When not consumed by FASN, malonyl CoA accumulates and allosterically inhibits CPT-1 activity to prevent fatty acid β-oxidation. Shutdown of this energy yielding process ultimately activates AMPK and promotes apoptosis [145]. These data are supported by the finding that TOFA, a compound that prevents carboxylation of acetyl-CoA to malonyl-CoA by inhibiting ACC, is not acutely cytotoxic in breast cancer cells despite preventing production of *de*

novo fatty acids [146]. Another study found that inhibition of ACC with Sorafen A can cause growth arrest, but that this could be prevented with exogenous palmitate, thereby indicating cell death was at least in part due to depletion of *de novo* fatty acids [147]. Moreover, it is clear that blocking the *synthesis* of fatty acids can also activate AMPK by disrupting the redox state and anaplerotic balance required to continue glycolytic energy production [148, 149]. Interestingly, it has been shown that the direct activation of CPT-1 by C75 is dominant to the inhibitory effect of malonyl-CoA accumulation to CPT-1. Therefore, the weight loss associated with C75 treatment cannot be avoided [139, 141].

Another drug, called orlistat (tetrahydrolipstatin), has been repositioned as a fatty acid synthase inhibitor. The drug's original application was for weight loss based on its inhibitory effects against gastric lipases thereby preventing fat absorption. Orlistat was later identified in a large screen for inhibitors of fatty acid synthase activity [150]. Although its use as a systemic fatty acid inhibitor with potential applications in cancer treatment is limited by a very poor bioavailability profile, a number of *in vitro* studies and animal models have demonstrated its efficacy as a fatty acid synthase inhibitor to reduce cancer cell growth [150, 151]. For example, intraperitoneal administration of orlistat reduced the size of established colorectal tumors in mice by 55 percent (P=.01) and PC-3 subcutaneous xenograft growth by 60% (P=.02) [150, 152]. One could envision using Orlistat to treat exposed cancers of the gastrointestinal tract with adequate bioavailability; however, there is evidence that long-term use of Orlistat in a mouse model can increase the risk of colorectal cancer potentially due to increased fecal fat excretion.

In addition, several families of plant-derived flavonoid compounds including epigallocatechin-3-gallate as well as luteolin and apigenin have been found to inhibit the

activity of FASN [153-155]. However, these compounds have notoriously high chemical reactivity and therefore their effects in the cell are less predictable. Similarly, the antibiotic cerulenin, produced by the fungus *Cephalosporium ceruleans*, is frequently used as a FASN inhibitor; however, it is known to be highly reactive in cells [156]. In fact, there is some evidence that it binds to cysteine residues in proteins and can block palmitoylation independent of FASN activity. Recently, an antibiotic produced by *Streptomyces platensis*, called platensimycin, was identified to inhibit fatty acid synthesis and, unlike the other inhibitors described, did not have any effect on sterol synthesis [157, 158]. Efforts to develop selective FASN inhibitors continue to be hampered by issues of chemical instability, binding promiscuity, and weak bioavailability [159]. Recent x-ray crystallography data for FASN will hopefully aid in the design of new targeted agents [160, 161].

Investigations into the Role of FASN in Cancer

Although there are still limitations with the FASN-targeted agents available, a great deal of work has been accomplished with them to better understand the relationship between FASN and cancer cell biology. An extensive review of the incidence and exploitability of FASN in cancer will follow.

Similar to prostate cancer, elevated expression of FASN is commonly detected in the earliest identifiable stages of colon cancer known as aberrant crypt foci (ACF) being detected in 86% of a 35 patient sample size [162]. In 1997, it was documented, by mincing colorectal carcinoma tissue samples and incubating with ^{14}C-acetate, that the lipogenic program was consistently hyperactive in this cancer type [163]. This was an

important study to show that, not only is FASN elevated, but it is producing excessive amounts of fatty acid in cancer.

FASN is upregulated in roughly half of all breast cancers [164]. Its expression in samples taken from primary surgical treatment of stage I breast carcinoma was found to be associated with a higher risk of cancer recurrence (P=.0001) and an overall poorer prognosis [122, 165]. In addition, elevated FASN contributes to drug resistance to Adriamycin and Mitoxantrone. Isolated subpopulations of resistant breast cancer cells were shown to have elevated FASN expression. Exogenous overexpression of FASN in the parental cell line could recapitulate the resistant phenotype, and inhibition of FASN activity could resensitize cells to these commonly used chemotherapeutic drugs. Most interestingly, exogenous palmitate (100 μM) alone was able to confer a level of resistance (1.3 fold change compared to a 2-3 fold change with FASN expression). The mechanism behind this resistance has not fully been understood, but, although plausible, did not seem to be through changes in membrane drug permeability or ABC multidrug transport systems. The authors of this report hypothesize that it was conferred potentially through changes in sphingosine and ceramide synthesis which are both downstream of FASN activity and involved in the mechanism of apoptosis induced by these chemotherapeutics [166]. An alternative theory is that FASN, or sufficient palmitate, allows the expression of prosurvival proteins, such as c-Met.

Pancreatic cancers are the 4th leading cause of cancer-related death in the U.S., and currently result in a 5-year survival rate of less than 5 percent. These cancers are highly resistant to radiation and chemotherapy. FASN expression correlates with poor prognosis, and in cell culture models FASN knockdown sensitizes cells to gemcitabine

and radiation treatments. These findings further establish a role for FASN in therapeutic resistance. Again, the authors of this study postulate that the mechanism of FASN-dependent resistance may involve a negative effect on ceramide-mediated apoptosis, but an alternative interpretation could be possible [167].

FASN expression is reported to be elevated in roughly 60% of diffuse large B-cell lymphomas (DLBCL) and is particularly high in proliferating cells (Ki67 staining) ($P<.0001$). Additionally in this study, the FASN inhibitor C75 was able to slow the growth of DLBCL cell lines (SUDHL5, 4, and 10) with a concomitant downregulation of c-Met protein [168]. This connection between FASN and c-Met was published subsequent to our laboratory's initial work that will be discussed in later chapters.

High FASN has been detected in precursor lesions of the colon, stomach, esophagus, oral cavity, prostate, and breast reemphasizing this is an early event in cancer development and suggesting applicability of chemopreventive FASN-targeting strategies. Accordingly, using the neu-N transgenic mouse model of mammary cancer, researchers showed that weekly treatments of the FASN inhibitor C247 reduced tumor incidence from 50% to 20%. C247 is a second-generation analogue of C75 that does not stimulate CPT-1, thereby limiting weight loss-associated toxicity [169]. These results suggest that, under certain cancer initiation conditions, FASN activity is required for development into frank cancer.

Oncogenic viruses are also influenced by FASN. Epstein-Barr virus (EBV) is associated with various cancers. It has been found that the immediate-early gene product BRLF1 upregulates FASN expression through a p38 MAPK pathway. This upregulation is required for lytic viral replication in human epithelial tongue cells. Similarly, B-cell

receptor engagement upregulates FASN expression and engagement of this receptor was required for induction of lytic replication in B-cells. It is not yet known why EBV lytic replication is dependent on FASN expression, but it is possible that lipid-derived signaling molecules or protein acylation is required. Alternatively, it is possible that a particular metabolic state is optimal for EBV replication [170]. Beyond EBV, replication of enteroviruses and members of the *Flaviviridae* family seem to require FASN expression possibly to maintain ER homeostasis and excess membrane biogenesis [171].

Thus far, a significant body of evidence has been presented to demonstrate the frequency with which FASN is observed at elevated levels in different cancers and the necessity of that expression in many cancers was revealed by the efficacy of FASN-targeting. It is also important to consider the possible reasons why FASN is commonly present and supposedly needed in cancer cells.

Given that FASN expression is known to be regulated by diet and energy balance; typically high carbohydrate, low fat diets increase FASN expression in bulk liver, smooth muscle, and some other tissue types such as colonic epithelium; the laboratories of Charles Fuchs and Massimo Loda investigated the effects of diet and FASN expression on colorectal cancer. These researchers showed that FASN expression can have a protective effect on 5-year cancer specific survival with 76% survival of FASN-negative patients and 96% survival of FASN-positive (P=.0002) in a cohort of 647 patients. The more interesting determination was that, when stratified, the protective effect was dependent on body mass index. The protective effect of elevated FASN was actually an increased risk in patients considered obese (BMI >27.5 kg/m^2). This report also cites similar unpublished results by the Nguyen laboratory with prostate cancer [172]. These

data emphasize the influence of host environment and the multifaceted context to which each molecular study must be considered. More specifically, one could speculate that colon cancer cells, or cancer cells in general, gain a significant growth/survival advantage by FASN upregulation through multiple possible mechanisms to be detailed in the coming sections. However, cells with upregulated FASN are more dependent on excess exogenous energy for growth; and therefore, individuals with low caloric intake and therefore low BMI starve the FASN-addicted tumors. Obese individuals have the excess calories the FASN-elevated tumors require to survive. Related to these findings, a recent report found that CD36 and LPL, proteins that facilitate uptake of circulating fatty acids, can, to some degree, substitute for the effects of FASN thereby suggesting a possible resistance mechanism to consider for future therapeutic strategies [173].

As solid tumors grow, areas of hypoxia universally develop. Hypoxia activates the PI3K and HIF1 pathway to upregulate SREBP1 gene expression and ultimately enzymes of the lipogenic phenotype. These findings further determined that hypoxia-induced chemoresistance is at least partly due to the upregulation of FASN. IHC analysis of breast cancer tissue shows FASN exceptionally high in regions of hypoxia [174]. FASN inhibition alone can cause an oxygen-independent hypoxia-like condition that dramatically (500-fold) increases the secretion of VEGF in Her2 overexpressing breast and ovarian cancer cells [175]. These results suggest caution is warranted when considering the use of FASN-targeting therapeutic strategies. They also suggest that fatty acid metabolism is a necessary metabolic adaptation to support the enhanced ability of Her2 cells to survive cellular hypoxia, because the lipogenic phenotype anaplerotically supports anaerobic glycolysis. When oxygen is limited, cells need to find oxidizing

power elsewhere. FASN activity consumes NADPH and; therefore, can aid in balancing the redox state providing NADP+ for continued glycolysis. In the absence of FASN, Her2 overexpressing cells are dependent on greater angiogenesis to survive [175]. The molecular connection between FASN and hypoxia is in agreement with the frequent detection of FASN in pre-malignant and early stages of cancer which are exceptionally hypoxic.

The endoplasmic reticulum (ER) is the major site of phospholipid biogenesis which is necessary for cell growth and proliferation. A subset of FASN resides in ER to provide free fatty acids for this process. Blocking FASN activity can cause a condition known as ER stress in highly proliferative cells, including cancer cells. The ER stress response will shutdown protein synthesis, promote protein degradation, and eventually induce cell death if membrane biogenesis cannot be restored [176]. This process could represent an additional susceptibility of cancer cells that can be exploited by targeting FASN.

Work by the laboratory of Ruth Lupu has identified a connection between the Her2 receptor tyrosine kinase and FASN. Using Orlistat to inhibit fatty acid synthase activity in the SK-BR3 breast cancer cell line, the researchers were able to determine that FASN activity maintained Her2 expression by negatively regulating the PEA3 transcriptional repressor. In the absence of FASN or its activity, PEA3 expression was increased repressing the expression of the important breast cancer oncogene, Her2. This was shown to result in cell-cycle arrest and apoptosis in the breast cancer cell line [164]. Conversely, overexpression of Her2 transcriptionally upregulates FASN expression in breast cancer cells through activation of the PI3K pathway [177]. Overexpression of

FASN can in turn hyperactivate EGFR family receptors through a mechanism possibly involving lipid raft composition [178]. Conversely, there is evidence that targeting FASN activity can disrupt lipid rafts to cause internalization and degradation of Her2 [179]. In fact, FASN inhibition and Her2 inhibition were found to be synergistically cytotoxic to breast and ovarian cancer cells [180].

The laboratories of Michael Freeman and Massimo Loda have shown that the expression of FASN is downstream of Caveolin-1, a lipid raft protein involved in receptor signaling and endocytic regulation. Caveolin-1 is a palmitoylated protein that their work has shown to physically complex with FASN transiently in lipid raft membrane domains. This interaction is induced by growth factors such as EGF that activate the Src and PI3K pathways. Palmitoylation-defective mutants of caveolin-1 did not activate downstream pathways and were not able to form complexes with FASN [181]. Previous work by this group along with Michael Lisanti, using prostate-specific caveolin-1 knockout mice in a TRAMP model of prostate cancer development, had already established caveolin-1 as essential to prostate cancer development and essential for the upregulation of FASN commonly observed in prostate cancer [182]. These results suggest a mechanism of reciprocal regulation by which signaling downstream of caveolin-1 upregulates FASN expression and caveolin-1 palmitoylation is regulated through relocalization of *de novo* palmitate synthesis. In a broader sense, this work is additional evidence that protein palmitoylation can be regulated by changes in FASN expression and/or localization. It also supports the notion that FASN-dependent control of acylation can have oncogenic effects.

CD44 is a transmembrane glycoprotein consistently elevated in colorectal cancer. There is some available evidence that c-Met requires association with CD44 for activity. A recent study showed that inhibition of FASN or ACLY lowered CD44 and reduced the associated c-Met signaling *in vitro* and *in vivo* [183]. This report was published after publication of the work detailed in this thesis, but it did not indicate as great a reduction in total c-Met protein. Differences in methodology involving available exogenous fatty acids could account for the discrepancies.

Excess fatty acids are primarily stored in cells as neutral fats, including triacylglycerols, diacylglycerols, and monoacylglycerols. Dietary fatty acids are primarily stored in stellate cells of the liver or specialized fibroblasts called adipocytes; however, cells with elevated FASN, such as cancer cells, may also need to store excess fatty acids. Research from the laboratory of Benjamin Cravatt has identified a lipolytic enzyme that seems to complement the role of *de novo* fatty acid synthesis in cancer. Newly synthesized fatty acids are either rapidly utilized or incorporated into neutral fats in the cell. This; therefore, necessitates a complementary lipolytic pathway to free stored fats at a later time. An activity-based proteomic analysis of hydrolytic enzymes in human cancer cells identified monoacylglycerol lipase (MAGL) as consistently elevated in aggressive cancer cells including melanoma, ovarian, and breast. MAGL hydrolyzes monoacylglycerols to free fatty acids including palmitate and stearate. Uniquely in cancer cells, MAGL was found to be necessary and sufficient to elevate free fatty acids, including palmitate, and this was able to confer increased tumorigenic and migratory properties [184]. It is interesting to consider these findings in the broader context of FASN expression and BMI with respect to cancer incidence, recurrence, and overall

prognosis as discussed throughout this literature review. There is overwhelming evidence that fatty acids are essential, but moreover, that they can drive cancer to a more aggressive phenotype which cancer cells then become dependent on or addicted to. Both the lipogenic pathway involving FASN and the lipolytic pathway involving MAGL would therefore seem to be attractive therapeutic targets, but there remains a great deal to learn about the distinctions between fatty acid sources, the balance between these seemingly redundant pathways, and the cancer-associated uses of free fatty acids.

A detailed study by Johannes Swinnen in the Verhoeven laboratory has provided insight into the unique role of cancer-associated FASN. Using shRNA to knockdown FASN expression, the researchers were able to analyze the contribution of FASN to the synthesis of distinct lipid classes in cancer cells. Lipid species were separated by thin-layer chromatography and analyzed for ^{14}C-acetate incorporation. It was determined that FASN-derived fatty acids are disproportionately processed into phospholipids, including phosphatidylcholine and phosphatidylserine, as opposed to cholesterol or triglycerides. Moreover, these *de novo* fatty acid-derived phospholipids selectively partitioned into detergent-resistant lipid-raft domains. This report added to the evidence that *de novo* fatty acids are probably utilized differently than dietary circulating fatty acids [185]. The experiments did not account for free fatty acids used for acylation of proteins. Interestingly, another group determined that inhibition of FASN can actually stimulate the oxidation of phospholipids into their component parts [186]. The saturation status of fatty acids can affect their organized alignment in membrane structures and thus can greatly alter integral and transversal dynamics. Using mass spectrometry-based phospholipid analysis, Swinnen et al. determined that clinical prostate tumor tissues with

elevated FASN have an increased degree of lipid saturation compared with non-lipogenic tumors. The ACC inhibitor Soraphen A or siRNA to FASN was able to decrease the concentration of saturated fatty acids in mouse xenografts and increase the amount of monounsaturated and polyunsaturated fatty acids. The authors explain that polyunsaturated fatty acids are more susceptible to peroxidation and therefore, low *de novo* lipogenesis leaves cancer cells more prone to oxidative-stress-induced cell death [187]. Also, considering saturated fatty acids are able to pack more densely in membrane structures, it is possible that this reduces the permeability of certain chemotherapeutic drugs.

FASN as a Mediator of Lipid Modifications

A recent study investigating the role of Wnt signaling in prostate cancer identified a correlation between elevated FASN expression and stabilized cytoplasmic β-catenin downstream of the active Wnt-signaling pathway. The authors explored this connection more closely by genetically manipulating FASN expression in non-cancerous prostate epithelial cells. It was determined, using ^{14}C-acetate labeling, that overexpression of FASN increased palmitoylation of Wnt-1 from FASN-derived palmitate and this was associated with active Wnt signaling and stabilized β-catenin [188]. This report is further evidence that *de novo* palmitate synthesis drives palmitoylation which can thereby regulate protein trafficking, stability, and activity.

An extensive study performed by the Swinnen laboratory, illustrates an intriguing role for lipogenesis in normal and cancer cell biology. The researchers mimicked the lipogenic phenotype in *Xenopus* embryos to observe changes during normal development. They observed major distortions in the microtubule sensory organelles

known as primary cilium. These minute membranous outreachings are present on the surface of most cells, but are lost in many cancers for unknown reasons. Reverting back to more versatile cell culture techniques, the authors found that inhibition of fatty acid synthesis in highly lipogenic prostate cancer cells actually restores to normal morphology. The most telling finding is that disruption of these structures is the result of total mislocalization of apical proteins and cell polarization, or in other words, a breakdown of normal subcellular architecture [189]. These findings suggest that hyperactive lipogenesis can, in and of itself, disrupt subcellular organization. It is reasonable to assume that unabated production of excess fatty acids causes unnecessary synthesis of membrane, unregulated acylation of proteins, and improper conversion of lipid signaling components. This speculation is exceptionally meaningful when questioning the oncogenic nature of FASN.

Review of Protein Acylation: Diversity, Techniques, and Influence

History of Protein Modifications with Fatty Acids

Protein acylation has become a well studied field over the last decade, due in large part to advances in technology and reagent availability. This post-translational modification was first detected over 30 years ago by Schlesinger and Schumidt at Washington University School of Medicine. Their initial discoveries found that radiolabeled palmitate could be covalently incorporated into viral membrane glycoproteins derived from cells infected with certain enveloped animal viruses [190]. Follow-up work by this same group determined that cellular proteins could also be covalently modified by the attachment of palmitate [191]. Work in this field over the next decade attempted to elucidate the properties acylation confers to a protein. The primary focus at the time was viral proteins due to the technical advantages of this work; however, work by Eric Olsen et al. began to explore acylation of cellular proteins and in particular the dynamics of this process. It was determined that the acylation of some proteins was responsive to different stimuli, whereas the acylation of other proteins seemed to be more stable [192]. The Olsen research group also observed the first suggestive evidence that there are spatial and temporal differences between the regulation and effects of distinct acyl groups [193].

The earliest identified forms of acylation involved palmitate or myristate; however, now isoprenyl groups, derived as intermediates in the HMG-CoA reductase-dependent cholesterol synthesis pathway, in the form of farensyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) are commonly detected modifications. Although technically considered a form of acylation, this later subgroup is often called prenylation

[194, 195]. In addition, the terms palmitoylation and acylation are frequently used interchangeably; however, in this work the word palmitoylation is used only when referring to the covalent attachment of the 16-carbon palmitate to a protein. Acylation will be used as a general term for post-translational modifications involving any lipid molecules. In general, post-translational modifications with acyl groups serve to add an increased level of hydrophobicity to proteins in order to direct membrane association. Although this is seemingly a crude adjustment to the chemical properties of a protein that would not be expected to provide a fine level of regulation, combinations and dynamics of acyl-group modifications can become a fine-tuned regulatory signal [196]. Several examples of regulation by acylation will be discussed here in detail to give a sense of the pleiotropic effects that have been observed, as well as an illustration of the significant void remaining in our understanding of this complex process.

Classes of Protein Acylation

There are limited reagents and techniques available for the study of protein acylation. For the first 20 years of research into the field of lipid protein-modifications, there were no drugs or compounds to inhibit these processes. The bulk of the work was done on crude protein purifications from cells incubated with radiolabeled lipids including: [^{3}H]myristate or palmitate, [^{3}H]GGPP or FPP, or their precursors [^{14}C]acetate or [^{14}C]mevalonate respectively. Eventually, site-directed mutagenesis allowed for the identification and manipulation of specific modified amino acid residues to help characterize the role of acylation for a particular protein. In 2000, the laboratory of Marilyn Resh at Sloan-Kettering published a report characterizing the first inhibitor of cellular palmitoylation [197]. The compound, 2-bromopalmitate (2-BP), irreversibly

inhibits general protein palmitoylation; however, the exact mechanism by which it acts is not fully accepted, but, regardless, it is widely used in the field as the standard palmitoylation inhibitor [198]. Subsequently a number of other acylation inhibitors became available including 2-hydroxymyristic acid (2-OHMyr) to inhibit myristoylation, as well as classes of specific farnesyltransferase inhibitors and geranylgeranyltransferase inhibitors. Development of prenylation inhibitors has developed more rapidly, and in fact, several have been tested in trials for clinical use, principally because the enzymes that catalyze prenylation are few and defined. Myristoylation is also catalyzed by a single enzyme, N-myristoyltranferase (NMT). Conversely, there are roughly two dozen enzymes encoded within the human genome that have a particular DHHC-cysteine rich motif characteristic of palmitoyl acyltranferases (PATs) [199, 200]. This makes the pairing of palmitoylated protein to its cognate PAT more difficult. Whereas the general inhibitory effect of 2-BP seems to cover all PAT activity, detailed studies of specific palmitoylated proteins may require the design of more specific inhibitors directed at particular PATs. Clearly, the identification of PAT to palmitoylated protein pairs would greatly aid the potential of future therapeutic usage. For example, in yeast, the protein Swf1 was determined to be responsible for palmitoylation of most, if not all, SNARE proteins at a single conserved juxtamembrane cysteine residue [201]. Research has uncovered some pairings like this, but most remain undetermined.

Palmitoylation seems to be unique from other forms of acylation in that it is typically a more dynamic process involving on and off signals at different subcellular membrane locations, thereby regulating proteins spatially and temporally. The depalmitoylation of proteins is; therefore, just as important as the palmitoylation.

Depalmitoylation is catalyzed by a smaller, less specific class of proteins termed acyl-protein thioesterases (APTs) [202]. Inhibitors of APTs are also being designed and tested for therapeutic benefit as they have been shown to affect the activity of many proteins, small GTPases in particular, involved in disease [202]. Another major difficulty with research on palmitoylated proteins is the lack of a strong consensus sequence to predict palmitoylation sites. Small GTPases often contain a sequence, known as the CAAX-box, which designates sites for prenylation and palmitoylation or myristoylation; however, the majority of palmitoylation sites seems to be haphazard as there is little apparent sequence homogeneity between proteins. In fact, some researchers in the field argue that palmitoylation in the absence of a detectable consensus sequence is likely to be the result of a nonenzymatic reaction [203]. Because of this, identification of palmitoylated cysteine residues often requires educated guessing and widespread mutagenesis of as many cysteine residues as possible.

In general, myristoylation occurs at an N-terminal glycine via a stable amide bond catalyzed by the aforementioned NMT; and is, more often than not, a co-translational event. Prenyl groups are stably linked to a cysteine residue via a thioester bond by their cognate transferase. Palmitoylation can attach via a stable amide bond to an N-terminal cysteine. This is a common modification of some secreted proteins like Wnt and Hedgehog and is catalyzed by a unique subset of PATS called membrane-bound O-acyltransferases (MBOAT) [202]. More commonly, palmitoylation occurs via a hydroxylamine-sensitive thioester linkage to a cysteine residue. The cysteine residues are usually either near a terminal end or adjacent to a transmembrane domain or peripherally membrane-associating polybasic sequence. Another difference is that transferases

responsible for prenylation or myristoylation are soluble cytoplasmic proteins often acting at early stages of protein biosynthesis and trafficking. PATs, conversely, seem to be consistently membrane associated and distributed more broadly throughout the cell. This broad distribution of PATs contributes to the ability of palmitoylation to be a more dynamic regulatory mechanism, whereas other lipid modifications seem to be a one-and-done event. Although the majority of PAT localization studies have been done in yeast, the results seem to parallel that which has been determined in human cells [204]

The functionality of most Rho family GTPases is controlled by subcellular distribution to localize activity. In large part, this level of control has been detailed in the work of Drs. Channing Der and Adrienne Cox. The seminal work by these two researchers discovered that most Rho family GTPases are lipid modified, and that this is dictated by a consensus sequence of a c-terminal cysteine followed by two aliphatic amino acids and a variable terminal amino acid (-CAAX). The cysteine in this motif is first either farnesylated or geranylgeranylated. This modification leads to removal of the -AAX peptide by endopeptidase cleavage and subsequent methylation of the prenylated cysteine. Removal of the -CAAX box from Rho GTPases prevents membrane association and causes accumulation of nonfunctional protein in the cytosol [205]. The requirement for these lipid modifications has been exploited in the development of several Rho GTPase inhibitors for clinical testing. This example also demonstrates the complexities of lipid modifications.

Novel Methods for the Identification of Acylated Proteins

More recently, alternative approaches have been developed to assay for palmitoylated proteins. The use of radiolabeled metabolites can slow progress due to the

relatively high levels of protein expression needed and the long exposure times often required. Although this can be overcome by ectopic expression of a tagged protein of interest, it is possible that supraphysiological expression of the palmitoylated protein can distort the reality of palmitoylation kinetics and regulation. A technique termed acyl-biotin exchange was developed in the last decade to allow for the detection of cysteine-modified native proteins. Immunoprecipitated proteins can be processed such that modified cysteine-linked lipid modifications can be swapped for biotin. Although this technique is relatively rapid and specific for cysteine modifications, it does not distinguish between different thioester-linked lipids [206]. In recent years, nonradiolabled lipid metabolites (lipid orthologs) have been developed, principally by Dr. Howard Hang, which can be used along with a Click-chemistry-based reaction to detect the incorporation of specific lipid species into proteins. Click-chemistry is a copper-catalyzed reaction that covalently links azide moieties to alkyne moieties. These moieties can be conjugated to metabolites such as palmitate as well as a means of detection such as biotin or a fluorophore [207, 208].

Acyl-biotin exchange and click-chemistry detection methods have both been used very recently for attempts at global palmitoyl proteomics studies in yeast, platelets, T- and B-cells, as well as endothelial cells [209-216]. These experiments have identified a number of previously unknown palmitoylated proteins, but primarily the same highly expressed dynamically palmitoylated are identified indicating significant limitations. The authors did not suggest that these proteomic studies were complete lists of all possible palmitoylated proteins. They conceded that there would be a substantial population of proteins that could be missed. If not expressed at high enough levels in the cell lines

examined or, for the click-based method, if turnover is limited, some proteins may be missed.

Using these techniques, a direct link between *de novo* fatty acid synthesis and palmitoylation has been demonstrated and, given the importance of this to the concordance of the thesis, details of these findings will be discussed.

A common feature of diabetes is severe gastrointestinal complications due to chronic inflammation of the colonic mucosa. FASN is a known insulin-responsive gene and is shutdown in the colonic epithelium in diabetes patients. Accordingly, Semenkovich et al. used Cre recombinase technology to selectively knockdown FASN expression in the colonic epithelium of mice to investigate a possible role for FASN in diabetes-associated colonic inflammation. Knockdown of FASN resulted in breakdown of the intestinal barrier similar to what is seen in diabetes. Ultimately it was determined that FASN knockdown prevents palmitoylation of Mucin-2, the major component of mucous secreted outside the colonic epithelium. The authors used an acyl-biotin exchange technique, which will be detailed later in this work, to monitor changes in palmitoylation of Mucin-2 [217]. Another study by this research group observed a similar example of FASN-regulated palmitoylation in the context of endothelial cell maintenance. Insulin is also known to influence vascular homeostasis, primarily through endothelial nitric-oxide synthase (eNOS). Maintenance of blood vessel integrity requires plasma membrane associated eNOS. The authors found that insulin-responsive FASN and eNOS physically associate at the plasma membrane and that this requires FASN-dependent palmitoylation of eNOS [218]. These reports do a thorough job of supporting

the concept that FASN expression alone can regulate protein palmitoylation *in vitro* and *in vivo*.

Palmitoylation: A Multifaceted Influence (Illustrative Examples)

The most interesting finding with respect to protein acylation has been the plethora of effects that lipid modifications can have – the major changes that subtle differences like the number, location, or species of lipids can have on a protein's trafficking, expression, and activity. Herein, a multitude of proteins and how they are affected by lipid modifications will be discussed. In keeping with the focus of this thesis, palmitoylation will be emphasized.

A recent report illustrates the difficulties associated with identifying palmitoylated proteins. The small GTPase Rac1 was first identified in 1989 and since then has become one of the most thoroughly studied proteins. Relatively early, Rac1 was determined to be post-translationally modified with a geranylgeranyl group via a CAAX-box and it was known to possess an extensive polybasic region, both of which could target to membrane. However, Rac1 was known to integrate into cholesterol-rich lipid raft microdomains and neither of these modifications have high affinity for such specific domains. Palmitoylation seems to have a higher affinity for these cholesterol-rich domains. In 2012, in order to make sense of this disconnect, Pozo et al. investigated the possibility that Rac1 was also palmitoylated. Using , [^{3}H]palmitate the researchers determined Rac1 is, in fact, palmitoylated at a single cysteine residue and nonpalmitoylated mutants were less active and did not partition into actin-linked lipid rafts [219]. The results came as a great surprise to a field that had studied this protein for decades.

The transferrin receptor is a single-transmembrane spanning protein that regulates transferrin-bound iron uptake. In 1981, the transferrin receptor became one of the earliest cellular proteins identified as palmitoylated, but the functional role of this was not established for a decade [220]. Kinetics experiments involving incorporation of [^{3}H] palmitate revealed that palmitoylation turnover at the plasma membrane regulates the rate of transferrin receptor internalization and recycling, and; therefore, the uptake of iron. Hormone and growth factor stimuli known to promote iron uptake were able to increase the amount of palmitoylated transferrin receptor by slowing its internalization thereby promoting surface expression [221]. Another example of a palmitoylated single-transmembrane spanning protein is the receptor tyrosine kinase low-affinity neurotrophic receptor (LNTR). LNTR is in the same protein family as tumor necrosis factor receptors (TNFRs), but bind specifically to nerve growth factor to maintain neuronal development. The receptor was found to be palmitoylated at a single cysteine residue within the intracellular juxtamembrane domain [222]. No follow-up work has been published to describe the influence of palmitoylation on LNTR.

One particular class of proteins found to consistently be regulated by palmitoylation is ion channels. The vast majority of these proteins are involved in neurotransmission and neuronal development, and thus, have major implications for mental health and motor neuron diseases [223]. NMDA receptors are a class of ionotropic glutamate receptors that function in excitatory neurotransmission and are formed from the coalescence of the NR1 and NR2 subunits. In a recent report by Huganir et al., the NR2 subunit was shown to be palmitoylated at a juxtamembrane region and a C-terminal region. Palmitoylation at the C-terminus gave an affinity for the

Golgi-apparatus, but the juxtamembrane palmitoylation promoted trafficking to and stability at the plasma membrane [224]. Similarly, all four subunits of another class of glutamate receptors, AMPA receptors, have been shown to get palmitoylated at two distinct sites, one in the transmembrane domain and another at the intracellular C-terminus. Palmitoylation in the transmembrane domain withholds the receptor in the Golgi, but palmitoylation at the c-terminus biases expression at the cell surface and regulates endocytosis [225]. These two examples seem to share a similar mechanism by which distinct palmitoylation domains regulate protein trafficking. It is likely that, in this scenario, the palmitoylated form of the protein is the default post-synthesis, and that *de*palmitoylation may be most influential on functional regulation. Consistent with this idea, the authors revealed that ligand-dependent activation of the AMPA receptor causes depalmitoylation, seemingly at both regions [225]. The ATP-gated cation channel, P2X7 receptor, is also palmitoylated at multiple cysteine residues. Palmitoylation defective mutants or 2BP treatment cause a substantial loss of surface expression and an overt accumulation within the ER. With time, this accumulated protein is slowly degraded through a lysosomal pathway [226].

Glutamic acid decarboxylase 65 (GAD65) is responsible for synthesis of the inhibitory neurotransmitter γ-amino butyric acid (GABA). GAD65 functions in a protein cluster at presynaptic membranes, but first must anchor to the cytoplasmic face of Golgi membranes. Interestingly, palmitoylation is not required for the initial Golgi-membrane association; this is conferred by an unknown hydrophobic modification, but palmitoylation of a single cysteine residue is required for exit from the Golgi and trafficking to the presynaptic membranes. More specifically, palmitoylation of GAD65

allows the protein to transition into cholesterol-rich membranes within the trans-Golgi secretory network which appropriately traffics the protein. Depalmitoylation has further been show to control levels of the enzyme at the synapse by promoting recycling back to the Golgi [227]. GABA receptors are heteropentamers resulting from the combination of 15 possible subunits. So far the γ2 subunit is the only one to be identified as palmitoylated. This subunit is, by far, the most frequently utilized subunit in GABA pentamer formation and its palmitoylation greatly increases the cell surface expression of the receptor [228]. A similar pentameric ligand-gated cation channel, the nicotinic acetylcholine receptor (nAChR), has been showed to get palmitoylated on multiple subunits. Palmitoylation deficiency in this receptor greatly reduced total protein nAChR expression, but increased the percent of the receptor present at the plasma membrane [229]. This possibly suggests a reduced stability during the trafficking process until a stable conformation is acquired at the cell surface.

The transport of dopamine into and out of the synaptic space between neurons is essential for proper motor activity and control of emotion. Dopamine transporter proteins (DATs) regulate the transport of dopamine from the synapse into the presynaptic neuron. In order to perform this function, DATs must be palmitoylated at a single cysteine near the intracellular side of its transmembrane domain. In the absence of palmitoylation, DATs are downregulated from the cell surface through proteasomal degradation thereby greatly reducing dopamine reuptake [230].

The G-protein coupled β1- and β2-adrenergic receptors are responsive to catecholamines and are facilitators of the fight-or-flight response, and as such regulate cardiac function and are primary targets for β-blocker therapy. These proteins were

determined to be palmitoylated based on a detailed understanding of their biosynthesis and trafficking which linked them with a known PAT. The cell surface expression of adrenergic receptors requires association with the Golgi protein golgin-160. Golgin-160 interacts with the Ras palmitoyltranferase. From this knowledge, the research groups of Howard Hang, Carolyn Machamer, and Yang Xiang, deduced that the trafficking of adrenergic receptors may be directed by palmitoylation in the Golgi. Using palmitate orthologs to detect palmitoylation, the authors were able to show that these receptors are palmitoylated. The β1-receptor was shown to have distinct clusters of palmitoylated cysteines that were regulated differently – one being a stable modification early in the biosynthetic pathway, whereas the other exhibited rapid turnover and regulated kinetics of internalization from the plasma membrane. Conversely, the β2-receptor is singly palmitoylated. Palmitoylation of this receptor is required for association with a signaling complex at the plasma membrane that promotes a localized protein kinase A (PKA) activity upon agonist binding. Components of PKA signaling are also kept within lipid rafts due to palmitoylation [231]. In the absence of β2 palmitoylation the receptor is downregulated normally upon ligand binding, but the entire complex is not internalized along with the receptor and therefore remains aberrantly active [232, 233].

The α subunit of G-protein coupled receptors is reversibly palmitoylated and this cycle of palmitoylation regulates its trafficking from the plasma membrane to the Golgi. At each membrane location, distinct DHHC PATs have been identified that influence the proteins palmitoylation status and thus regulate its shuttling [234].

Protease-activated receptor 2 (PAR2) was determined, using palmitate-ortholog labeling, to require palmitoylation for expression at the cell surface. PAR2 is

proteolytically cleaved at the plasma membrane in response to agonist. This cleavage leads to internalization and degradation. Interestingly, agonist-induced cleavage increases the palmitoylation kinetics of nascent PAR2 at a post-ER site . The researchers identified the site of palmitoylation by using brefeldin A treatment to block transport of protein from the ER to the Golgi. This treatment prevented PAR2 palmitoylation indicating it is stably palmitoylated after exit from the ER, and through an unknown mechanism, agonist treatment increases the rate of this process [235]. Considering PAR2 at the surface is rapidly cleaved and internalized in response to agonist, it is likely that agonist stimulates the palmitoylation of PAR2 stored in the Golgi until a fresh pool of PAR2 is required at the plasma membrane.

Interferon-α (IFN-α) Receptor 1 (IFNAR1) is activated by the ligand (IFN-α) as an immunomodulatory signal that subsequently activates the downstream Jak/STAT signaling pathway. IFNAR1 was determined to be palmitoylated, and preventing palmitoylation, either pharmacologically or genetically, diminished its ability to activate STAT1/2. Interestingly, this was despite seemingly normal intracellular distribution and expression; however, the kinetics of ligand-stimulated endocytosis was slowed, possibly explaining the decrease in downstream signaling [236].

Often thought of as merely a cytoskeletal protein straightforwardly regulated by nucleation, branching, and polymerization; tubulin is actually post-translationally modified to a great extent. Tubulin is regulated by acetylation, phosphorylation, tyrosination as well as palmitoylation. Palmitoylation of tubulin occurs at multiple cysteine residues in its monomeric form, but these modifications are thought to persist, at

least in part, in the polymerized form to provide increased hydrophobicity for the intercalation of segments into membrane structures [237-239].

Cav-1, as discussed previously, is a membrane associated protein that defines a subset of lipid raft domains and controls signaling and endocytosis of certain cell surface proteins. It was also discussed that Cav-1 associates with FASN through a mechanism that may promote its functionally required palmitoylation. Palmitoylation is known to occur at a single cysteine residue near its membrane-spanning domain, and this is required for it to be phosphorylated by the tyrosine kinase Src. Interestingly, Src is lipid-modified by myristoylation to target it, seemingly, to the same membrane domain where it interacts with Cav-1 [240].

Extensive research in the laboratory of Marilyn Resh has demonstrated that most of the Src-family kinases are themselves palmitoylated; however, Src is not palmitoylated. For example, the Src-family kinase Lyn requires palmitoylation for trafficking to the plasma membrane through Golgi exocytic secretory vesicles. Lyn does not appear to localize at particular membrane domains, in fact, palmitoylation excluded this kinase from concentrating at focal adhesions. Mutating the palmitoylation site of Lyn caused the protein to traffic in a manner indistinguishable from Src. Nonpalmitoylated Lyn reached the plasma membrane through the Golgi, but rather than resting at the plasma membrane, it cycled to and from late endosomes and, under particular stimuli, intercalated into focal adhesions. These data are compelling evidence of the critical influence a single lipid group can confer to a protein, in essence making it a completely new protein [241]. Typically; however, most other Src family kinases including Fyn, Lck, and LAT are similarly directed to detergent resistant membranes by

palmitoylation as exhibited in T-cells and several epithelial cell lines [197]. Of note, LAT requires palmitoylation for exit from the Golgi and trafficking to the plasma membrane. Inhibition of palmitoylation reduced the protein's half-life through targeted proteasomal degradation [242].

The G-protein coupled chemokine receptor CCR5 is expressed on several lymphoid and myeloid cell types including T- and B-cells as well as macrophages and dendritic cells. In fact, CCR5 is the principle coreceptor for HIV binding and internalization on macrophages. CCR5 requires palmitoylation for efficient surface expression, and if blocked by 2-BP treatment or cysteine mutagenesis, the receptor accumulates along the biosynthetic pathway without escaping the Golgi [243].

Lysosomes are highly regulated organelles responsible for protein degradation and recycling in a cell. Directing newly synthesized lysosomal proteins to their site of function is the responsibility of the mannose-6-phosphate receptor (M6PR). This receptor, along with sortilin, binds to and partitions off proteins destined for lysosomes. These proteins bud out of the Golgi into clathrin-coated endosomes that will fuse with lysosomes. M6PR releases the cargo in the endosomes and recycles back to the Golgi associated with the retromer complex. This highly ordered process is regulated by dynamic palmitoylation of M6PR and sortilin. Palmitoylation-defective mutants are unable to associate with the retromer, and; therefore, accumulate in endosomes and become degraded in the lysosomes [244].

The nuclear hormone receptor estrogen receptor (ER)α, localizes to the plasma membrane, but upon ligand binding translocates into the nucleus to directly function as a transcription factor for estrogen-responsive genes. In addition to its DNA-binding

function, ERα activates downstream signaling pathways including PI3K/Akt and Erk. ERα localizes to Cav-1 positive membrane domains in its palmitoylated form. Palmitoylated ERα constitutively activates a basal level of downstream signaling, whereas, binding of its ligand, 17-β-estradiol, causes depalmitoylation, internalization, and translocation into the nucleus for genomic activity [245, 246]. An additional report found that preventing proper integration of ERα into Cav-1-positive membrane domains by blocking its palmitoylation promotes proteasomal degradation as opposed to nuclear transport in response to ligand [247]. This major consequence illustrated the importance of lipid modifications for accurate protein targeting into specific membrane domains.

Tetraspanins are members of a large family of multipass transmembrane proteins that play roles in everything from neuronal development to viral fusion. The members of this family are palmitoylated in a Golgi compartment, but data suggest it is not required for spatially regulated function. It does, however, affect stability and therefore half-life of the protein [248].

As mentioned in yeast, most members of the SNARE family of membrane fusion regulators are known to be palmitoylated. In particular, the member SNAP25 is palmitoylated at 4 cysteine residues which are each modified independently giving an elevated level of calibration. Cycles of palmitoylation and depalmitoylation at the plasma membrane, recycling endosomes, and trans-Golgi vesicles regulate the affinity of SNAP25 and thus the fusion and differentiation of distinct intracellular vesicles. The dynamic regulation of SNAP25 was determined using cycloheximide to prove that levels of palmitoylation were changing independent of new protein entering the SNAP25 pool [249]. Similarly, syntaxin 7 and syntaxin 8 that function in endosomal fusion are

regulated by palmitoylation. Syntaxin 7 and 8 are palmitoylated in the ER and post-Golgi vesicles respectively, as indicated by palmitoylation kinetics experiments in the presence or absence of brefeldin A [250].

The yeast chitin synthase protein (Chs3) requires palmitoylation to exit the ER. Preventing palmitoylation of Chs3 caused the proteins to form aggregates within the ER; therefore it was proposed that palmitoylation caused the protein to adopt a stable confirmation that allows ER exit [251]. This role for palmitoylation is in contrast to the membrane targeting role more commonly attributed to lipid modifications; however, it seems highly likely that lipid modifications also cause conformation changes to the protein structure which would have an equally important function.

The general effects of long term global palmitoylation inhibition, particularly with 2-BP treatment, have also been described. One common result seems to be the induction of ER stress, due to an excessive protein load within this organelle after significant incubation periods. A prolonged block of palmitoylation apparently caused a number of proteins to accumulate in the ER not being able to take on the proper conformation required for exit from the ER [252]. These results, in and of themselves, suggest that a large population of proteins require palmitoylation early along the biosynthetic pathway to attain an appropriate conformation for maintained expression, stability, and proper trafficking.

Wnt and Hedgehog proteins are known to be palmitoylated, but the consequence was only recently elucidated. Hannoush et al. determined the subcellular distribution of the palmitoylated forms of each of these proteins using palmitate orthologs and click-chemistry detection. It was determined that palmitoylated forms only localize to cellular

membrane fractions [253]. The secreted ligand sonic hedgehog (Shh) influences embryonic development. Work by the Resh laboratory has found that Shh is palmitoylated at an N-terminal cysteine and this is required for the protein to form into soluble multimeric complexes in order to function as potent a ligand [254]. This is an example of the rare incidence in which a palmitoylated protein acts as a soluble secreted polyprotein, suggested that lipid modifications are not only important for membrane association.

The mechanics behind anthrax toxin binding in and entry into cells has been well characterized. The toxin is composed of three polypeptides, one of which binds directly to one of two cell surface receptors, either the tumor endothelial marker 8 or capillary morphogenesis gene 2, which will be collectively called anthrax receptors (ARs). Upon toxin binding, it was known that the ARs must translocate into lipid rafts where they can be ubiquitinated and internalized with the toxin. Although palmitoylation is frequently shown to promote protein partitioning into lipid raft domains, Goot et al. recently showed that palmitoylation prevents ARs from entering lipid rafts. This suggests, in the context of toxin binding, there is a depalmitoylation event that promotes translocation into rafts, though this has not be definitely shown. The authors do experimentally determine that ARs are initially palmitoylated early along the biosynthetic, but that there is significant turnover that occurs at a post-Golgi location [255]. Again, this demonstrates the irregular effects of protein palmitoylation.

Wrch-1 is a Wnt-regulated CDC42 homolog of the Rho GTPase family. Comparing Wrch-1 with its homolog CDC42 provides some interesting insight into the role lipid modifications play in conferring distinct function to similar proteins. Wrch-1

has a unique C-terminal extension in its polybasic region, but both homologs terminate with a CAAX-box. In CDC42, this CAAX-box directs prenylation; however, because of this subtle difference, Wrch-1 is not prenylated but palmitoylated. The different lipid species dictate entirely disparate subcellular distribution. CDC42 is distributed throughout the cytoplasm and commonly found perinuclear, but the palmitoylated Wrch-1 localizes exclusively to endosomes and the plasma membrane. Wrch-1 overexpression in cells conferred transforming potential and this is completely blocked when its spatially regulated function was blocked by palmitoylation-deficient mutations [256]. Similarly, the small GTPases H- and N-Ras are palmitoylated, but K-Ras is not. The peripheral association of K-Ras to membranes seems to be entirely dependent on a long polybasic region. In addition to the GTPases themselves, it makes sense that the activating guanine nucleotide exchange factors (GEFs) would need to be similarly localized, and accordingly, several GEFs have been identified as palmitoylated proteins. For example, p63RhoGEF is directed to the plasma membrane by palmitoylation [257].

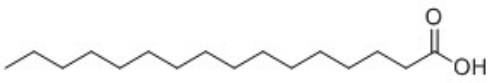
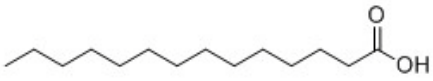
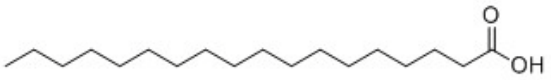
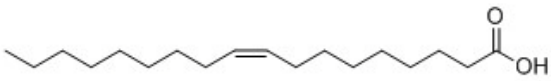
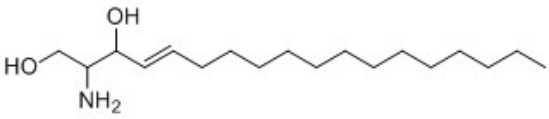
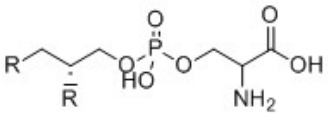
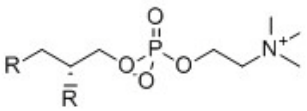
The γ-secretase complex is an integral plasma membrane proteolytic complex that has a role in receptor shedding and is implicated in the processing of amyloid proteins associated with Alzheimer's disease. There are four protein subunits that make up the complex. The subunits nicastrin and APH-1 are palmitoylated. It has been shown that palmitoylation is not required for the complex to form at the plasma membrane, but that it is required for the complex to intercalate into detergent-resistant lipid raft domains. The data from this report indicated that the half-life of newly synthesized palmitoylation defective nicastrin or APH-1 was significantly reduced before it complexes [258]. Another study found that the γ-secretase complex, in conjunction with the ADAM10

protease, caused betacellulin ectodomain shedding and the formation of a remnant intracellular fragment. The betacellulin ectodomain fragment acts as a ligand for the EGFR family receptors ErbB1 and ErbB4, whereas the intracellular fragment can associate with the nuclear membrane and arrest cell growth. The activity of the intracellular fragment seems to be regulated by palmitoylation. Inhibiting palmitoylation reduces the level of fragment formation [259].

This extensive discussion of palmitoylation's role in cell biology was aimed at emphasizing the diversity of responses to palmitoylation and the subtleties that influence this response. Herein, palmitoylation has been shown to be required for egress from the ER, maturation through the Golgi, cycling between the Golgi and endocytic vesicles, essential conformational changes, and maintained stability and expression of distinct proteins under distinct conditions. Moreover, these reports serve as precedence for the techniques used and interpretations made in this thesis.

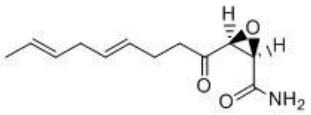
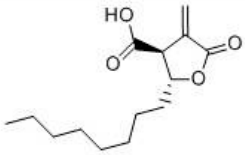
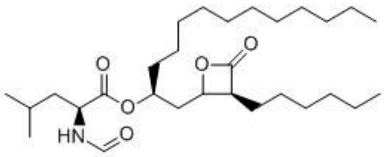
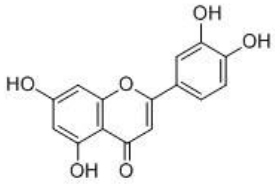
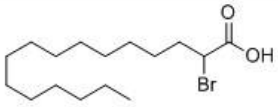
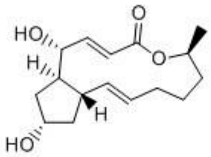
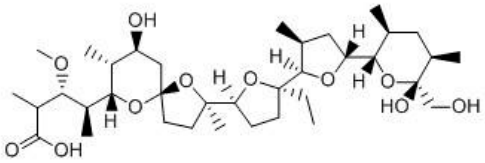
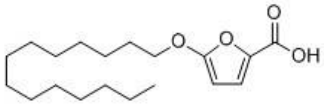
Literature Review Table

Table 1.

Palmitic Acid		16-carbon saturated
Myristic Acid		14-carbon saturated
Stearic Acid		18-carbon saturated
Oleaic Acid		18-carbon monounsaturated
Sphingosine		18-carbon amino alcohol unsaturated chain
Phosphatidylserine		Phospholipid R: fatty acid chain
Phosphatidylcholine		Phospholipid R:fatty acid chain

Literature Review Table

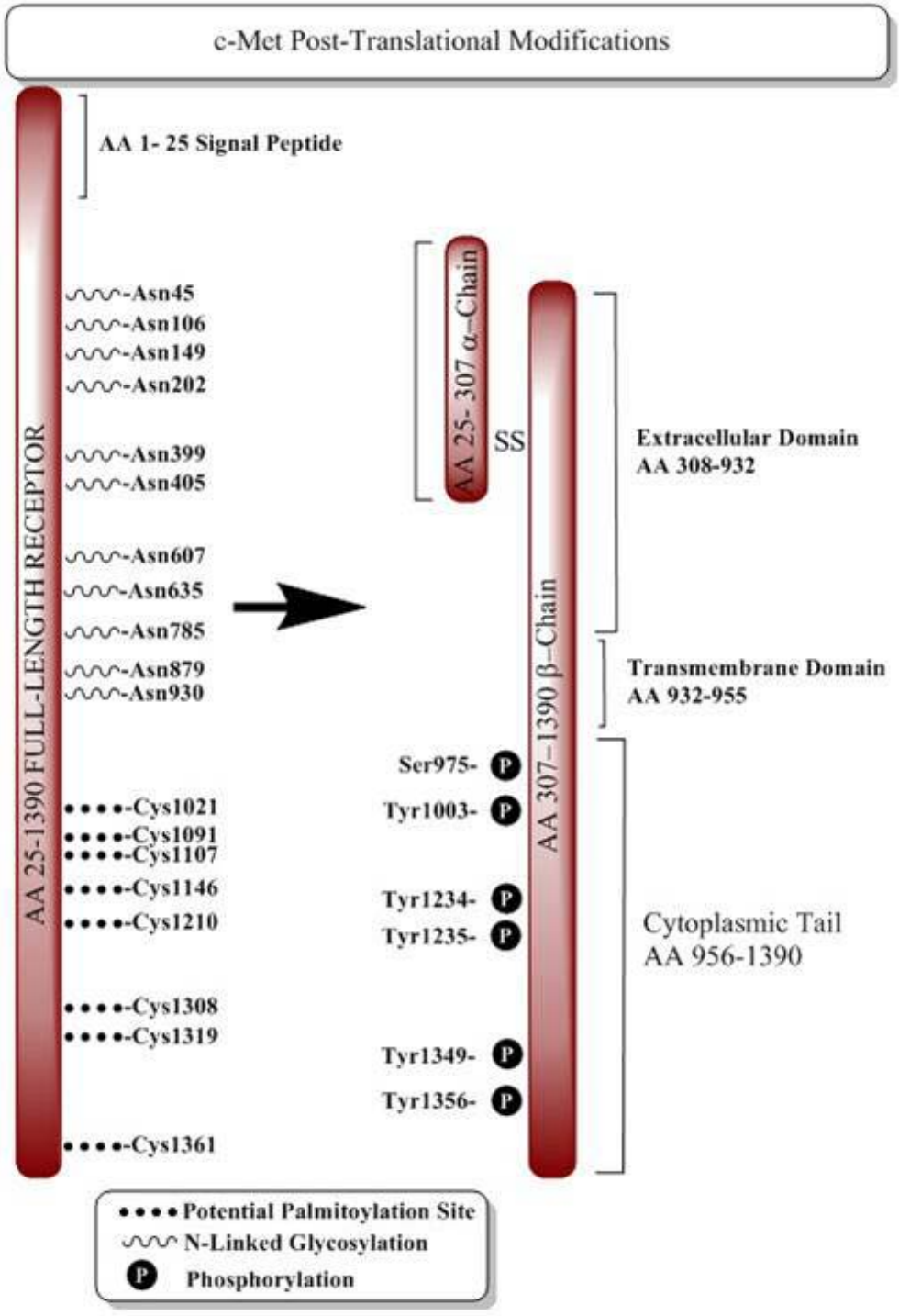
Table 2.

Cerulenin		Nonspecific FASN Inhibitor
C75		FASN Inhibitor
Orlistat		Nonspecific FASN Inhibitor
Luteolin		Nonspecific FASN Inhibitor
2-Bromopalmitate		Palmitoylation Inhibitor
Brefeldin A		Inhibitor of ER to Golgi Anterograde Transport
Monensin		Inhibitor of Anterograde Protein Transport From the Golgi
TOFA		Inhibitor of Acetyl-CoA Carboxylase

Literature Review Figure Legend

Figure 1. Schematic of c-Met processing and modifications. The c-Met protein is synthesized as a 170 kd single chain precursor that is heavily modified in the ER by N-linked glycosylation at asparagine residues. There are numerous cysteine residues along the cytoplasmic tail and within the juxtamembrane domain that have the potential to be palmitoylated. The precursor protein is proteolytically processed in the Golgi into a disulfide-linked α chain (50 kd) and β chain (140 kd) linked by disulfide bonds. Multiple tyrosine and serine residues have been shown to become phospohrylated in response to dimerization.

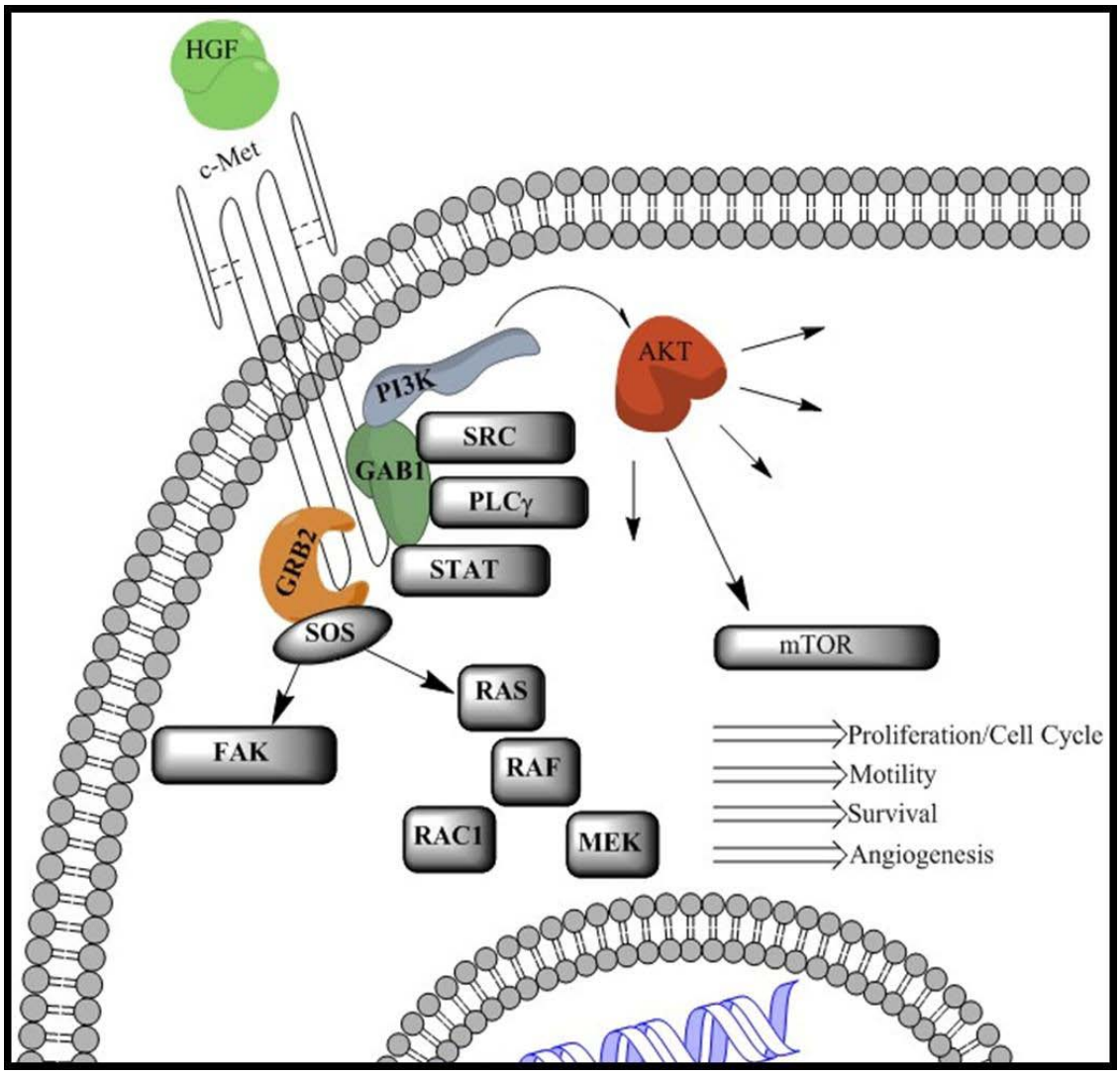
Literature Review Figure



Literature Review Figure Legend

Figure 2. The c-Met signaling axis. c-Met dimerizes in response to binding of its ligand hepatocyte growth factor (HGF). Dimerization initiates a phosphorylation cascade that recruits adaptor proteins to facilitate activation of downstream signaling pathways including those of the Src-family kinases, signal transducer and activator of transcription (STAT), phospholipase C (PLC), phosphoinositide-3-kinase (PI3K), mammalian target of rapamycin (mTOR), focal adhesion kinase (FAK) as well as Ras and mitogen-activated protein kinase (MAPK). These signaling pathways culminate in changes to the cell cycle, motility and invasion, survival, and angiogenesis through regulation of gene expression, protein turnover and activity, and actin rearrangements.

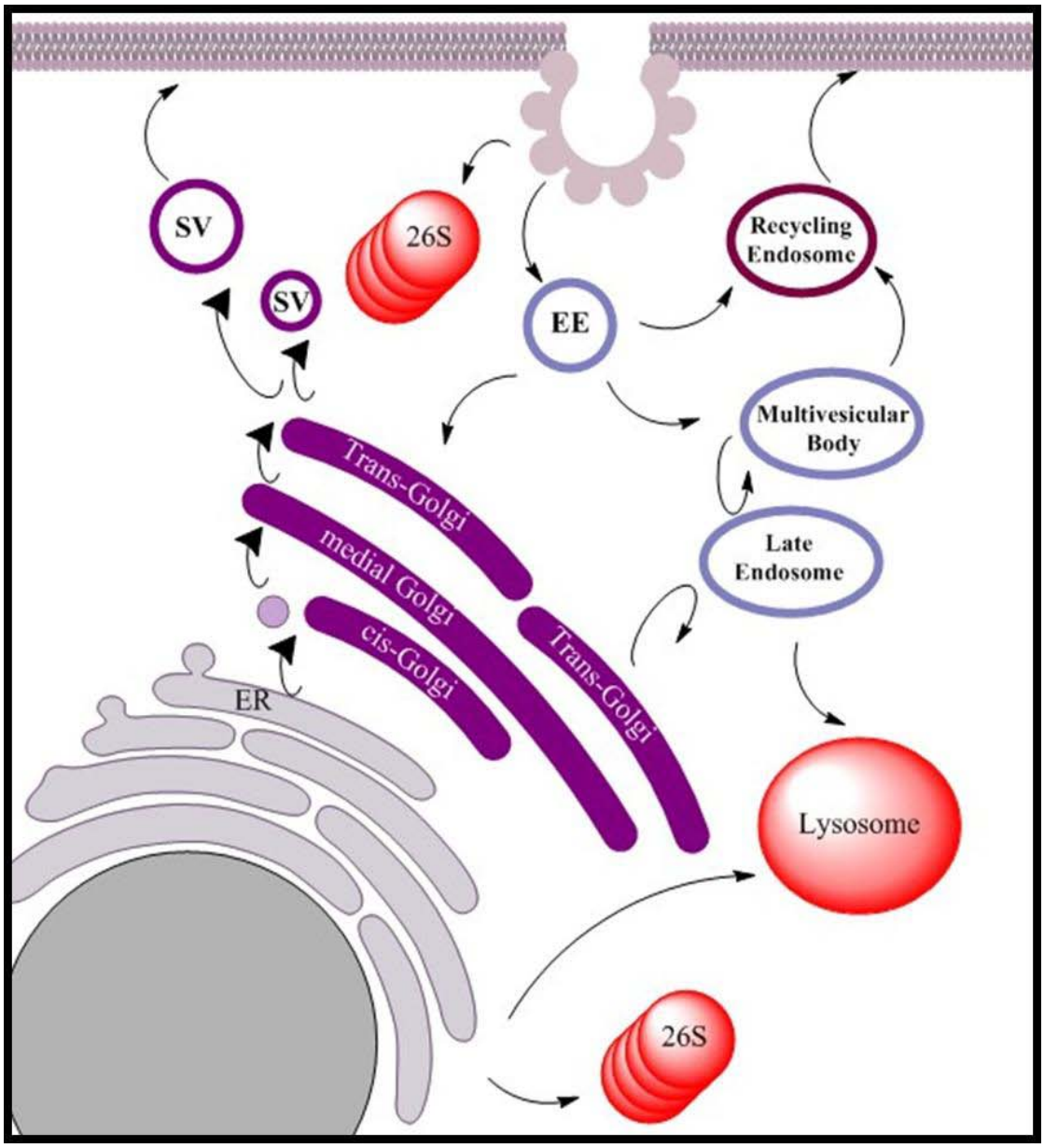
Literature Review Figure



Literature Review Figure Legend

Figure 3. Schematic of general protein trafficking. Integral membrane mRNA, including that of type I single-spanning transmembrane proteins such as c-Met, are translated into the ER via a signal sequence (N-terminal for c-Met) recognized by the signal recognition particle (SRP) at the ER surface. The initial translated sequence is directed into the ER through the translocon and entry is halted at the transmembrane domain as it is appropriately oriented spanning the ER bilayer. Proteins can then be post-translationally processed by proteolysis, glycosylation, or acylation. Proteins destined for expression at the plasma membrane are shuttled from the ER to the Golgi and ultimately sorted into secretory vesicles (SV). Mechanisms regulating the sorting of different proteins toward distinct subcellular locations are highly convoluted and not fully understood. Upon fusion of SVs with the plasma membrane, transmembrane proteins are retained at the membrane while soluble proteins are secreted. Integrated into the plasma membrane, cell surface expression can be downregulated by internalization. There are numerous mechanisms of vesicular internalization including clathrin or caveolae mediated, and bulk macropinocytosis. The internalized vesicles are termed early endosomes (EE) which may be routed through many possible paths to ultimately degrade or recycle the vesicle contents. Degradation can occur by fusion with lysosomes or by delivery to the proteasome (26S).

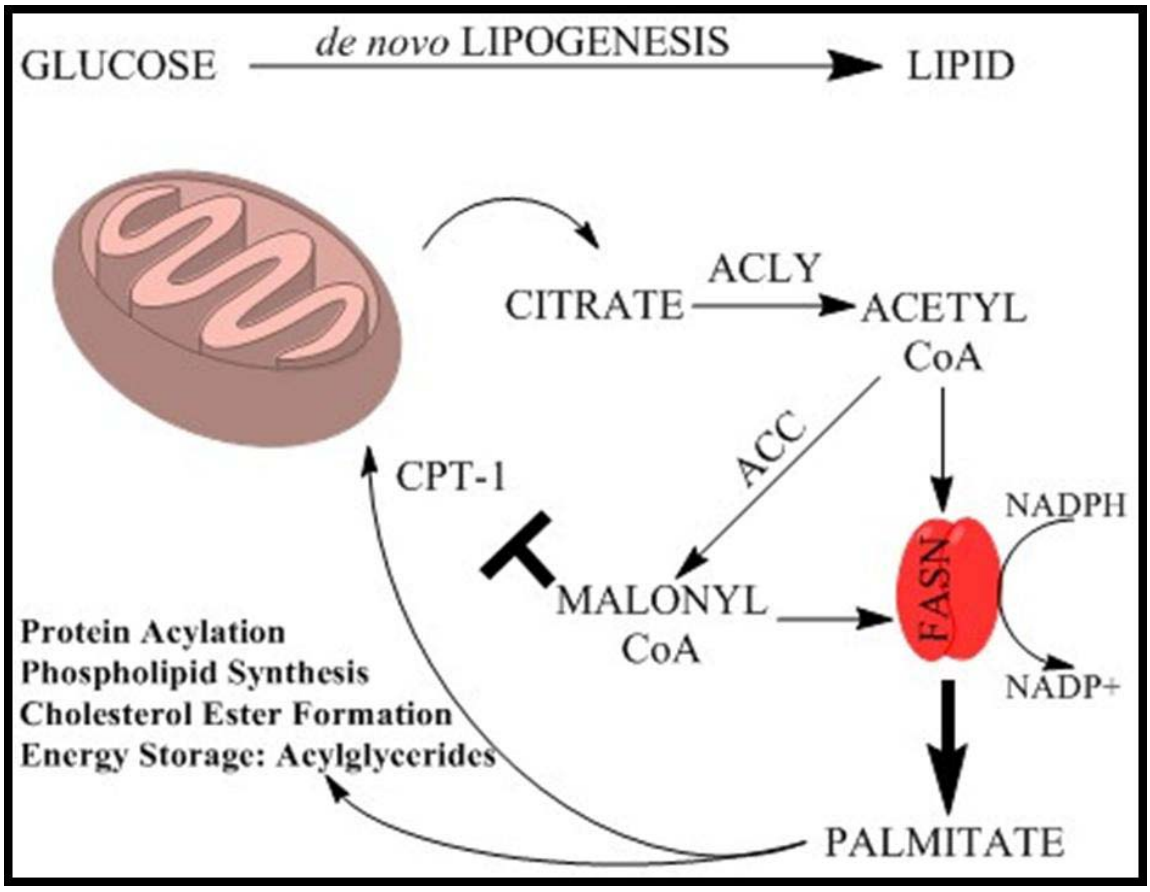
Literature Review Figure



Literature Review Figure Legend

Figure 4. Fatty acid metabolism. Fatty acid synthase (FASN) predominately catalyzes the synthesis of the 16-carbon saturated fatty acid palmitate from the condensation of 2-carbon donor malonyl CoA to the single carbon primer acetyl CoA. ATP citrate lyase (ACLY) converts citrate shunted from mitochondria into acetyl CoA. A portion of acetyl CoA is converted to malonyl CoA by acetyl CoA carboxylase (ACC) in the rate-limiting step. Palmitate can be used for the acylation of proteins, esterified to phospholipids for incorporation into the plasma membrane, stored for future ATP requirements, or to form cholesterol esters for integration of cholesterol into plasma membrane and for storage. Carnitine palmitoyltransferase (CPT)-1 is responsible for the transport of palmitate into mitochondria for β-oxidation to release stored energy. During a high energy state, CPT-1 is kept inhibited by excess malonyl CoA.

Literature Review Figure



References:

1. Cooper, C.S., et al., Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature, 1984. **311**(5981): p. 29-33.
2. Park, M., et al., Mechanism of met oncogene activation. Cell, 1986. **45**(6): p. 895-904.
3. Giordano, S., et al., Tyrosine kinase receptor indistinguishable from the c-met protein. Nature, 1989. **339**(6220): p. 155-6.
4. Prat, M., et al., C-terminal truncated forms of Met, the hepatocyte growth factor receptor. Mol. Cell. Biol., 1991. **11**(12): p. 5954-5962.
5. Giordano, S., et al., Biosynthesis of the protein encoded by the c-met proto-oncogene. Oncogene, 1989. **4**(11): p. 1383-8.
6. Crepaldi, T., et al., Generation of a truncated hepatocyte growth factor receptor in the endoplasmic reticulum. J Biol Chem, 1994. **269**(3): p. 1750-5.
7. Gherardi, E., et al., Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12039-44.
8. Guo, A., et al., Signaling networks assembled by oncogenic EGFR and c-Met. Proceedings of the National Academy of Sciences, 2008. **105**(2): p. 692-697.
9. Duhon, D., et al., The polyphenol epigallocatechin-3-gallate affects lipid rafts to block activation of the c-Met receptor in prostate cancer cells. Mol Carcinog, 2010. **49**(8): p. 739-49.
10. Lu, Y.C. and H.C. Chen, Involvement of lipid rafts in adhesion-induced activation of Met and EGFR. J Biomed Sci, 2012. **18**(2012): p. 78.
11. Giordano, S., et al., p145, a protein with associated tyrosine kinase activity in a human gastric carcinoma cell line. Mol Cell Biol, 1988. **8**(8): p. 3510-7.
12. Naldini, L., et al., The tyrosine kinase encoded by the MET proto-oncogene is activated by autophosphorylation. Mol Cell Biol, 1991. **11**(4): p. 1793-803.

13. Ferracini, R., et al., Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. J Biol Chem, 1991. **266**(29): p. 19558-64.

14. Hartmann, G., et al., A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis. Proc Natl Acad Sci U S A, 1992. **89**(23): p. 11574-8.

15. Stoker, M., et al., Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature, 1987. **327**(6119): p. 239-42.

16. Weidner, K., et al., Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J. Cell Biol., 1990. **111**(5): p. 2097-2108.

17. Weidner, K.M., et al., Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7001-5.

18. Naldini, L., et al., Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. Embo J, 1992. **11**(13): p. 4825-33.

19. Trusolino, L. and P.M. Comoglio, Scatter-factor and semaphorin receptors: cell signalling for invasive growth. 2002. **2**(4): p. 289-300.

20. Rong, S., et al., Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. Mol Cell Biol, 1992. **12**(11): p. 5152-8.

21. Birchmeier, C., et al., Met, metastasis, motility and more. Nat Rev Mol Cell Biol, 2003. **4**(12): p. 915-925.

22. Rosario, M. and W. Birchmeier, How to make tubes: signaling by the Met receptor tyrosine kinase. Trends Cell Biol, 2003. **13**(6): p. 328-35.

23. Tulasne, D., et al., The multisubstrate docking site of the MET receptor is dispensable for MET-mediated RAS signaling and cell scattering. Mol Biol Cell, 1999. **10**(3): p. 551-65.

24. Trusolino, L., A. Bertotti, and P.M. Comoglio, MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol, 2010. **11**(12): p. 834-48.

25. Bowers, D.C., et al., Scatter Factor/Hepatocyte Growth Factor Protects against Cytotoxic Death in Human Glioblastoma via Phosphatidylinositol 3-Kinase- and AKT-dependent Pathways. Cancer Res, 2000. **60**(15): p. 4277-4283.

26. Xu, J., et al., Effect of Akt inhibition on scatter factor-regulated gene expression in DU-145 human prostate cancer cells. Oncogene, 2007. **26**(20): p. 2925-38.

27. Tulasne, D. and B. Foveau, The shadow of death on the MET tyrosine kinase receptor. 2007. **15**(3): p. 427-434.

28. Bosse, T., et al., Cdc42 and Phosphoinositide 3-Kinase Drive Rac-Mediated Actin Polymerization Downstream of c-Met in Distinct and Common Pathways. Mol. Cell. Biol., 2007. **27**(19): p. 6615-6628.

29. Chen, S.-Y. and H.-C. Chen, Direct Interaction of Focal Adhesion Kinase (FAK) with Met Is Required for FAK To Promote Hepatocyte Growth Factor-Induced Cell Invasion. Mol. Cell. Biol., 2006. **26**(13): p. 5155-5167.

30. Chen, T.H., et al., Phosphorylation of focal adhesion kinase on tyrosine 194 by Met leads to its activation through relief of autoinhibition. Oncogene. **30**(2): p. 153-66.

31. Jensen, A.R., et al., Fyn is downstream of the HGF/MET signaling axis and affects cellular shape and tropism in PC3 cells. Clin Cancer Res, 2011. **17**(10): p. 3112-22.

32. Boccaccio, C., et al., Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature, 1998. **391**(6664): p. 285-8.

33. Kermorgant, S. and P.J. Parker, Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol, 2008. **182**(5): p. 855-63.

34. Steffan, J.J., et al., HGF-induced invasion by prostate tumor cells requires anterograde lysosome trafficking and activity of Na+-H+ exchangers. J Cell Sci. **123**(Pt 7): p. 1151-9.

35. Steffan, J.J., et al., Na+/H+ Exchangers and RhoA Regulate Acidic Extracellular pH-Induced Lysosome Trafficking in Prostate Cancer Cells. Traffic, 2009. **10**(6): p. 737-753.

36. Cantelmo, A.R., et al., Cell delivery of Met docking site peptides inhibit angiogenesis and vascular tumor growth. Oncogene, 2010. **29**(38): p. 5286-98.

37. Lim, J. and J.P. Thiery, Epithelial-mesenchymal transitions: insights from development. Development, 2012. **139**(19): p. 3471-86.

38. Kaldenbach, M., et al., Hepatocyte growth factor/c-Met signalling is important for the selection of transplanted hepatocytes. Gut, 2012. **61**(8): p. 1209-18.

39. Kunio Matsumoto, T.N., Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. International Journal of Cancer, 2006. **119**(3): p. 477-483.

40. Boccaccio, C. and P.M. Comoglio, Invasive growth: a MET-driven genetic programme for cancer and stem cells. Nat Rev Cancer, 2006. **6**(8): p. 637-45.

41. Gambarotta, G., et al., Structure and inducible regulation of the human MET promoter. J Biol Chem, 1994. **269**(17): p. 12852-7.

42. Pennacchietti, S., et al., Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell, 2003. **3**(4): p. 347-361.

43. Carmen Eckerich, S.Z., Regina Fillbrandt, Sonja Loges, Manfred Westphal, Katrin Lamszus,, Hypoxia can induce c-Met expression in glioma cells and enhance SF/HGF-induced cell migration. International Journal of Cancer, 2007. **121**(2): p. 276-283.

44. Morozov, V.M., et al., Regulation of c-met expression by transcription repressor Daxx. 2007. **27**(15): p. 2177-2186.

45. Li, Y., et al., Elevated expression of eukaryotic translation initiation factor 4E is associated with proliferation, invasion and acquired resistance to erlotinib in lung cancer. cbt, 2012. **13**(5

1538-4047): p. 272-280.

46. Webb, C.P., et al., The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. Cancer Res, 2000. **60**(2): p. 342-9.

47. Gandino, L., et al., Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. J Biol Chem, 1994. **269**(3): p. 1815-20.

48. Hashigasako, A., et al., Bi-directional regulation of Ser-985 phosphorylation of c-met via protein kinase C and protein phosphatase 2A involves c-Met activation and cellular responsiveness to hepatocyte growth factor. J Biol Chem, 2004. **279**(25): p. 26445-52.

49. Kakazu, A., G. Sharma, and H.E. Bazan, Association of protein tyrosine phosphatases (PTPs)-1B with c-Met receptor and modulation of corneal epithelial wound healing. Invest Ophthalmol Vis Sci, 2008. **49**(7): p. 2927-35.

50. Lai, A.Z., et al., Met kinase-dependent loss of the E3 ligase Cbl in gastric cancer. J Biol Chem, 2012. **287**(11): p. 8048-59.

51. Peschard, P., et al., Mutation of the c-Cbl TKB Domain Binding Site on the Met Receptor Tyrosine Kinase Converts It into a Transforming Protein. Molecular Cell, 2001. **8**(5): p. 995-1004.

52. Peschard, P., et al., A conserved DpYR motif in the juxtamembrane domain of the Met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required for suppression of oncogenic activation. J Biol Chem, 2004. **279**(28): p. 29565-71.

53. Petrelli, A., et al., The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met. Nature, 2002. **416**(6877): p. 187-90.

54. Li, N., et al., Specific Grb2-mediated interactions regulate clathrin-dependent endocytosis of the cMet-tyrosine kinase. J Biol Chem, 2007. **282**(23): p. 16764-75.

55. Raiborg, C., T.E. Rusten, and H. Stenmark, Protein sorting into multivesicular endosomes. Curr Opin Cell Biol, 2003. **15**(4): p. 446-55.

56. Hammond, D.E., et al., Endosomal Dynamics of Met Determine Signaling Output. Mol. Biol. Cell, 2003. **14**(4): p. 1346-1354.

57. Mosesson, Y., G.B. Mills, and Y. Yarden, Derailed endocytosis: an emerging feature of cancer. 2008. **8**(11): p. 835-850.

58. Parachoniak, C.A. and M. Park, Dynamics of receptor trafficking in tumorigenicity. Trends Cell Biol, 2012. **22**(5): p. 231-40.

59. Lefebvre, J., et al., Met degradation: more than one stone to shoot a receptor down. Faseb J, 2012. **26**(4): p. 1387-99.

60. Chaudhuri, A., et al., Distinct involvement of the Gab1 and Grb2 adaptor proteins in signal transduction by the related receptor tyrosine kinases RON and MET. J Biol Chem, 2011. **286**(37): p. 32762-74.

61. Kermorgant, S., D. Zicha, and P.J. Parker, Protein Kinase C Controls Microtubule-based Traffic but Not Proteasomal Degradation of c-Met. J. Biol. Chem., 2003. **278**(31): p. 28921-28929.

62. Jeffers, M., et al., Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol. Cell. Biol., 1997. **17**(2): p. 799-808.

63. Hammond, D.E., et al., Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene, 2001. **20**(22): p. 2761-70.

64. Carter, S., S. Urbe, and M.J. Clague, The Met Receptor Degradation Pathway. J. Biol. Chem., 2004. **279**(51): p. 52835-52839.

65. Hoffmann, K.M., et al., Gastrointestinal Hormones Cause Rapid c-Met Receptor Down-regulation by a Novel Mechanism Involving Clathrin-mediated Endocytosis and a Lysosome-dependent Mechanism. J. Biol. Chem., 2006. **281**(49): p. 37705-37719.

66. Abella, J.V., et al., Dorsal ruffle microdomains potentiate Met receptor tyrosine kinase signaling and down-regulation. J Biol Chem, 2011. **285**(32): p. 24956-67.

67. Joffre, C., et al., A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol, 2011. **13**(7): p. 827-37.

68. Parachoniak, C.A., et al., GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. Dev Cell, 2012. **20**(6): p. 751-63.

69. Kermorgant, S. and P.J. Parker, c-Met signalling: spatio-temporal decisions. Cell Cycle, 2005. **4**(3): p. 352-5.

70. Tulasne, D., et al., Proapoptotic function of the MET tyrosine kinase receptor through caspase cleavage. Mol Cell Biol, 2004. **24**(23): p. 10328-39.

71. Foveau, B., et al., Amplification of apoptosis through sequential caspase cleavage of the MET tyrosine kinase receptor. Cell Death Differ, 2007. **14**(4): p. 752-64.

72. Nath, D., et al., Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. J Cell Sci, 2001. **114**(6): p. 1213-1220.

73. Petrelli, A., et al., Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. Proc Natl Acad Sci U S A, 2006. **103**(13): p. 5090-5.

74. Schirrmeister, W., et al., Ectodomain shedding of E-cadherin and c-Met is induced by Helicobacter pylori infection. Exp Cell Res, 2009. **315**(20): p. 3500-8.

75. Wajih, N., J. Walter, and D.C. Sane, Vascular origin of a soluble truncated form of the hepatocyte growth factor receptor (c-met). Circ Res, 2002. **90**(1): p. 46-52.

76. Foveau, B., et al., Down-regulation of the met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. Mol Biol Cell, 2009. **20**(9): p. 2495-507.

77. Ancot, F., et al., Shedding-generated Met receptor fragments can be routed to either the proteasomal or the lysosomal degradation pathway. Traffic, 2012. **13**(9): p. 1261-72.

78. Athauda, G., et al., c-Met ectodomain shedding rate correlates with malignant potential. Clin Cancer Res, 2006. **12**(14 Pt 1): p. 4154-62.

79. Mazzone, M. and P.M. Comoglio, The Met pathway: master switch and drug target in cancer progression. FASEB J., 2006. **20**(10): p. 1611-1621.

80. Peters, S. and A.A. Adjei, MET: a promising anticancer therapeutic target. Nat Rev Clin Oncol, 2012. **9**(6): p. 314-26.

81. Watanabe, M., et al., Progression-linked overexpression of c-Met in prostatic intraepithelial neoplasia and latent as well as clinical prostate cancers. Cancer Letters, 1999. **141**(1-2): p. 173-178.

82. Gastaldi, S., P.M. Comoglio, and L. Trusolino, The Met oncogene and basal-like breast cancer: another culprit to watch out for? Breast Cancer Res, 2010. **12**(4): p. 208.

83. Knudsen, B.S., et al., High expression of the Met receptor in prostate cancer metastasis to bone. Urology, 2002. **60**(6): p. 1113-1117.

84. Jeffers, M., et al., The mutationally activated Met receptor mediates motility and metastasis. Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(24): p. 14417-14422.

85. Jeffers, M., et al., Activating mutations for the Met tyrosine kinase receptor in human cancer. Proceedings of the National Academy of Sciences, 1997. **94**(21): p. 11445-11450.

86. Jeffers, M.F., Activating mutations in the Met receptor overcome the requirement for autophosphorylation of tyrosines crucial for wild type signaling. Oncogene, 1999. **18**(36): p. 5120-5.

87. Cappuzzo, F., et al., Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol, 2009. **27**(10): p. 1667-74.

88. Mak, H.H.L., et al., Oncogenic activation of the Met receptor tyrosine kinase fusion protein, Tpr-Met, involves exclusion from the endocytic degradative pathway. 2007. **26**(51): p. 7213-7221.

89. Wickramasinghe, D. and M. Kong-Beltran, Met activation and receptor dimerization in cancer: a role for the Sema domain. Cell Cycle, 2005. **4**(5): p. 683-5.

90. Lee, J.H., et al., An alternatively spliced form of Met receptor is tumorigenic. Exp Mol Med, 2006. **38**(5): p. 565-73.

91. Hill, K.S., M. Lorinczi, and L.A. Elferink, Altered down regulation of the receptor tyrosine kinase met in pancreatic adenocarcinoma cells. J Exp Ther Oncol, 2010. **8**(4): p. 297-312.

92. Danilkovitch-Miagkova, A. and B. Zbar, Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. J Clin Invest, 2002. **109**(7): p. 863-7.

93. Abella, J.V., et al., Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol, 2005. **25**(21): p. 9632-45.

94. Kentsis, A., et al., Autocrine activation of the MET receptor tyrosine kinase in acute myeloid leukemia. Nat Med, 2012. **18**(7): p. 1118-22.

95. Tyan, S.W., et al., Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. PLoS One, 2011. **6**(1): p. e15313.

96. Gherardi, E., et al., Targeting MET in cancer: rationale and progress. Nat Rev Cancer, 2012. **12**(2): p. 89-103.

97. Christensen, J.G., J. Burrows, and R. Salgia, c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett, 2005. **225**(1): p. 1-26.

98. Steffan, J.J., D.T. Coleman, and J.A. Cardelli, The HGF-met signaling axis: emerging themes and targets of inhibition. Curr Protein Pept Sci, 2011. **12**(1): p. 12-22.

99. De Bacco, F., et al., The MET oncogene is a functional marker of a glioblastoma stem cell subtype. Cancer Res, 2012. **72**(17): p. 4537-50.

100. van Leenders, G.J., et al., Activation of c-MET induces a stem-like phenotype in human prostate cancer. PLoS One, 2012. **6**(11): p. e26753.

101. Joo, K.M., et al., MET signaling regulates glioblastoma stem cells. Cancer Res, 2012. **72**(15): p. 3828-38.

102. Gastaldi, S., et al., Met signaling regulates growth, repopulating potential and basal cell-fate commitment of mammary luminal progenitors: implications for basal-like breast cancer. Oncogene, 2012.

103. Scheel, C. and R.A. Weinberg, Cancer stem cells and epithelial mesenchymal transition: Concepts and molecular links. Seminars in Cancer Biology, 2012. **22**(5â€“6): p. 396-403.

104. Yano, S., et al., Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. Cancer Res, 2008. **68**(22): p. 9479-87.

105. Chen, C.T., et al., MET activation mediates resistance to lapatinib inhibition of HER2-amplified gastric cancer cells. Mol Cancer Ther, 2012. **11**(3): p. 660-9.

106. Turke, A.B., et al., Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell, 2012. **17**(1): p. 77-88.

107. Rho, J.K., et al., The role of MET activation in determining the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. Mol Cancer Res, 2009. **7**(10): p. 1736-43.

108. Tanizaki, J., et al., Differential roles of trans-phosphorylated EGFR, HER2, HER3, and RET as heterodimerisation partners of MET in lung cancer with MET amplification. Br J Cancer, 2011. **105**(6): p. 807-13.

109. Engelman, J.A., et al., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science, 2007. **316**(5827): p. 1039-43.

110. Shattuck, D.L., et al., Met Receptor Contributes to Trastuzumab Resistance of Her2-Overexpressing Breast Cancer Cells. Cancer Res, 2008. **68**(5): p. 1471-1477.

111. Straussman, R., et al., Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. 2012. **487**(7408): p. 500-504.

112. Wilson, T.R., et al., Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. 2012**.** advance online publication.

113. Catenacci, D.V., et al., Durable complete response of metastatic gastric cancer with anti-Met therapy followed by resistance at recurrence. Cancer Discov, 2012. **1**(7): p. 573-9.

114. Kuhajda, F.P., Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. Nutrition, 2000. **16**(3): p. 202-208.

115. Kuhajda, F.P., Fatty Acid Synthase and Cancer: New Application of an Old Pathway. Cancer Res, 2006. **66**(12): p. 5977-5980.

116. Watt, M.J., et al., Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. Am J Physiol Endocrinol Metab, 2012. **302**(1): p. E1-3.

117. Menendez, J.A., et al., Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu oncogene amplification. Ann Oncol, 2005. **16**(3): p. 359-71.

118. Menendez, J.A., R. Colomer, and R. Lupu, Inhibition of fatty acid synthase-dependent neoplastic lipogenesis as the mechanism of gamma-linolenic acid-induced toxicity to tumor cells: an extension to Nwankwo's hypothesis. Med Hypotheses, 2005. **64**(2): p. 337-41.

119. Chirala, S.S., et al., Fatty acid synthesis is essential in embryonic development: fatty acid synthase null mutants and most of the heterozygotes die in utero. Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6358-63.

120. Daye, D. and K.E. Wellen, Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. Semin Cell Dev Biol, 2012. **23**(4): p. 362-9.

121. Menendez, J.A. and R. Lupu, Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer, 2007. **7**(10): p. 763-777.

122. Kuhajda, F.P., et al., Fatty acid synthesis: a potential selective target for antineoplastic therapy

Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(14): p. 6379-6383.

123. Kuhajda, F.P., S. Piantadosi, and G.R. Pasternack, Haptoglobin-related protein (Hpr) epitopes in breast cancer as a predictor of recurrence of the disease. N Engl J Med, 1989. **321**(10): p. 636-41.

124. Milgraum, L.Z., et al., Enzymes of the fatty acid synthesis pathway are highly expressed in in situ breast carcinoma. Clin Cancer Res, 1997. **3**(11): p. 2115-20.

125. Prowatke, I., et al., Expression analysis of imbalanced genes in prostate carcinoma using tissue microarrays. 2006. **96**(1): p. 82-88.

126. Swinnen, J.V., et al., Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. International Journal of Cancer, 2002. **98**(1): p. 19-22.

127. Rossi, S., et al., Fatty Acid Synthase Expression Defines Distinct Molecular Signatures in Prostate Cancer. Mol Cancer Res, 2003. **1**(10): p. 707-715.

128. Shah, U.S., et al., Fatty acid synthase gene overexpression and copy number gain in prostate adenocarcinoma. Human Pathology, 2006. **37**(4): p. 401-409.

129. Swinnen, J.V., et al., Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. Oncogene, 2000. **19**(45): p. 5173-81.

130. Lee, M.Y., et al., KLF5 enhances SREBP-1 action in androgen-dependent induction of fatty acid synthase in prostate cancer cells. Biochem J, 2009. **417**(1): p. 313-22.

131. Lucy J. Schmidt, K.V.B., Donald J. Tindall,, Inhibition of fatty acid synthase activity in prostate cancer cells by dutasteride. The Prostate, 2007. **67**(10): p. 1111-1120.

132. Bandyopadhyay, S., et al., FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. Oncogene, 2005. **24**(34): p. 5389-5395.

133. Tine Van de Sande, T.R., Evelyne Lerut, Steven Joniau, Hein Van Poppel, Guido Verhoeven, Johannes V Swinnen,, High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. The Journal of Pathology, 2005. **206**(2): p. 214-219.

134. De Schrijver, E., et al., RNA Interference-mediated Silencing of the Fatty Acid Synthase Gene Attenuates Growth and Induces Morphological Changes and Apoptosis of LNCaP Prostate Cancer Cells. Cancer Res, 2003. **63**(13): p. 3799-3804.

135. Wang, H.Q., et al., Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene, 2005. **24**(22): p. 3574-3582.

136. Van de Sande, T., et al., Role of the Phosphatidylinositol 3'-Kinase/PTEN/Akt Kinase Pathway in the Overexpression of Fatty Acid Synthase in LNCaP Prostate Cancer Cells. Cancer Res, 2002. **62**(3): p. 642-646.

137. Porstmann, T., et al., PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. 2005. **24**(43): p. 6465-6481.

138. Migita, T., et al., Fatty Acid Synthase: A Metabolic Enzyme and Candidate Oncogene in Prostate Cancer. J. Natl. Cancer Inst., 2009. **101**(7): p. 519-532.

139. Thupari, J.N., et al., C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9498-502.

140. Cha, S.H., et al., Inhibition of hypothalamic fatty acid synthase triggers rapid activation of fatty acid oxidation in skeletal muscle. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(41): p. 14557-14562.

141. Kim, E.-K., et al., C75, a Fatty Acid Synthase Inhibitor, Reduces Food Intake via Hypothalamic AMP-activated Protein Kinase. Journal of Biological Chemistry, 2004. **279**(19): p. 19970-19976.

142. Bentebibel, A., et al., Novel effect of C75 on carnitine palmitoyltransferase I activity and palmitate oxidation. Biochemistry, 2006. **45**(14): p. 4339-50.

143. Orita, H., et al., Selective Inhibition of Fatty Acid Synthase for Lung Cancer Treatment. Clinical Cancer Research, 2007. **13**(23): p. 7139-7145.

144. Orita, H., et al., Inhibiting Fatty Acid Synthase for Chemoprevention of Chemically Induced Lung Tumors. Clinical Cancer Research, 2008. **14**(8): p. 2458-2464.

145. Zhou, W., et al., Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. Cancer Res, 2007. **67**(7): p. 2964-71.

146. Pizer, E.S., et al., Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. Cancer Res, 2000. **60**(2): p. 213-8.

147. Beckers, A., et al., Chemical inhibition of acetyl-CoA carboxylase induces growth arrest and cytotoxicity selectively in cancer cells. Cancer Res, 2007. **67**(17): p. 8180-7.

148. Oliveras-Ferraros, C., et al., AMPK-sensed cellular energy state regulates the release of extracellular Fatty Acid Synthase. Biochem Biophys Res Commun, 2009. **378**(3): p. 488-93.

149. Pike, L.S., et al., Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. Biochim Biophys Acta, 2010. **1807**(6): p. 726-34.

150. Kridel, S.J., et al., Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity. Cancer Res, 2004. **64**(6): p. 2070-2075.

151. Carvalho, M.A., et al., Fatty acid synthase inhibition with Orlistat promotes apoptosis and reduces cell growth and lymph node metastasis in a mouse melanoma model. Int J Cancer, 2008. **123**(11): p. 2557-65.

152. Chuang, H.Y., Y.F. Chang, and J.J. Hwang, Antitumor effect of orlistat, a fatty acid synthase inhibitor, is via activation of caspase-3 on human colorectal carcinoma-bearing animal. Biomed Pharmacother, 2011. **65**(4): p. 286-92.

153. Menendez, J.A., et al., Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays. Int J Mol Med, 2008. **22**(4): p. 433-9.

154. Puig, T., et al., Green tea catechin inhibits fatty acid synthase without stimulating carnitine palmitoyltransferase-1 or inducing weight loss in experimental animals. Anticancer Res, 2008. **28**(6A): p. 3671-6.

155. Brusselmans, K., et al., Induction of Cancer Cell Apoptosis by Flavonoids Is Associated with Their Ability to Inhibit Fatty Acid Synthase Activity. J. Biol. Chem., 2005. **280**(7): p. 5636-5645.

156. Vazquez, M.J., et al., Discovery of GSK837149A, an inhibitor of human fatty acid synthase targeting the beta-ketoacyl reductase reaction. Febs J, 2008. **275**(7): p. 1556-67.

157. Wang, J., et al., Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature, 2006. **441**(7091): p. 358-61.

158. Wu, M., et al., Antidiabetic and antisteatotic effects of the selective fatty acid synthase (FAS) inhibitor platensimycin in mouse models of diabetes. Proc Natl Acad Sci U S A, 2011. **108**(13): p. 5378-83.

159. Lupu, R., R. Colomer, and J.A. Menendez, An easy, rapid and objective mathematical method to identify fatty acid synthase (oncogenic antigen-519) modulators with potential anticancer value. Clin Transl Oncol, 2008. **10**(4): p. 219-26.

160. Asturias, F.J., et al., Structure and molecular organization of mammalian fatty acid synthase. Nat Struct Mol Biol, 2005. **12**(3): p. 225-32.

161. Maier, T., S. Jenni, and N. Ban, Architecture of mammalian fatty acid synthase at 4.5 A resolution. Science, 2006. **311**(5765): p. 1258-62.

162. Kearney, K.E., T.G. Pretlow, and T.P. Pretlow, Increased expression of fatty acid synthase in human aberrant crypt foci: possible target for colorectal cancer prevention. Int J Cancer, 2009. **125**(1): p. 249-52.

163. Rashid, A., et al., Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. Am J Pathol, 1997. **150**(1): p. 201-8.

164. Menendez, J.A., L. Vellon, and R. Lupu, Antitumoral actions of the anti-obesity drug orlistat (XenicalTM) in breast cancer cells: blockade of cell cycle progression, promotion of apoptotic cell death and PEA3-mediated transcriptional repression of Her2/neu (erbB-2) oncogene. Ann Oncol, 2005. **16**(8): p. 1253-67.

165. Alo, P.L., et al., Expression of fatty acid synthase (FAS) as a predictor of recurrence in stage I breast carcinoma patients. Cancer, 1996. **77**(3): p. 474-82.

166. Liu, H., Y. Liu, and J.T. Zhang, A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction. Mol Cancer Ther, 2008. **7**(2): p. 263-70.

167. Yang, Y., et al., Role of fatty acid synthase in gemcitabine and radiation resistance of pancreatic cancers. Int J Biochem Mol Biol, 2011. **2**(1): p. 89-98.

168. Uddin, S., et al., Inhibition of fatty acid synthase suppresses c-Met receptor kinase and induces apoptosis in diffuse large B-cell lymphoma. Mol Cancer Ther, 2010. **9**(5): p. 1244-55.

169. Alli, P.M., et al., Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. Oncogene, 2005. **24**(1): p. 39-46.

170. Li, Y., et al., Fatty acid synthase expression is induced by the Epstein-Barr virus immediate-early protein BRLF1 and is required for lytic viral gene expression. J Virol, 2004. **78**(8): p. 4197-206.

171. Martin-Acebes, M.A., et al., West Nile virus replication requires fatty acid synthesis but is independent on phosphatidylinositol-4-phosphate lipids. PLoS One, 2011. **6**(9): p. e24970.

172. Ogino, S., et al., Cohort study of fatty acid synthase expression and patient survival in colon cancer. J Clin Oncol, 2008. **26**(35): p. 5713-20.

173. Kuemmerle, N.B., et al., Lipoprotein lipase links dietary fat to solid tumor cell proliferation. Mol Cancer Ther, 2011. **10**(3): p. 427-36.

174. Furuta, E., et al., Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1. Cancer Res, 2008. **68**(4): p. 1003-1011.

175. Menendez, J.A., et al., Does endogenous fatty acid metabolism allow cancer cells to sense hypoxia and mediate hypoxic vasodilatation? Characterization of a novel molecular connection between fatty acid synthase (FAS) and hypoxia-inducible factor-1alpha (HIF-1alpha)-related expression of vascular endothelial growth factor (VEGF) in cancer cells overexpressing her-2/neu oncogene. J Cell Biochem, 2005. **94**(5): p. 857-63.

176. Little, J.L., et al., Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells. Cancer Res, 2007. **67**(3): p. 1262-9.

177. Kumar-Sinha, C., et al., Transcriptome Analysis of HER2 Reveals a Molecular Connection to Fatty Acid Synthesis. Cancer Res, 2003. **63**(1): p. 132-139.

178. Vazquez-Martin, A., et al., Overexpression of fatty acid synthase gene activates HER1/HER2 tyrosine kinase receptors in human breast epithelial cells. Cell Proliferation, 2008. **41**(1): p. 59-85.

179. Menendez, J.A., L. Vellon, and R. Lupu, Targeting fatty acid synthase-driven lipid rafts: a novel strategy to overcome trastuzumab resistance in breast cancer cells. Medical Hypotheses, 2005. **64**(5): p. 997-1001.

180. Menendez, J.A., et al., Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(29): p. 10715-10720.

181. Di Vizio, D., et al., Caveolin-1 interacts with a lipid raft-associated population of fatty acid synthase. Cell Cycle, 2008. **7**(14): p. 2257-67.

182. Di Vizio, D., et al., Caveolin-1 is required for the upregulation of fatty acid synthase (FASN), a tumor promoter, during prostate cancer progression. Cancer Biol Ther, 2007. **6**(8): p. 1263-8.

183. Zaytseva, Y.Y., et al., Inhibition of fatty acid synthase attenuates CD44-associated signaling and reduces metastasis in colorectal cancer. Cancer Res, 2012. **72**(6): p. 1504-17.

184. Nomura, D.K., et al., Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. Cell, 2010. **140**(1): p. 49-61.

185. Swinnen, J.V., et al., Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochemical and Biophysical Research Communications, 2003. **302**(4): p. 898-903.

186. Zhou, W., et al., Fatty Acid Synthase Inhibition Triggers Apoptosis during S Phase in Human Cancer Cells. Cancer Res, 2003. **63**(21): p. 7330-7337.

187. Rysman, E., et al., De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Res, 2010. **70**(20): p. 8117-26.

188. Fiorentino, M., et al., Overexpression of fatty acid synthase is associated with palmitoylation of Wnt1 and cytoplasmic stabilization of beta-catenin in prostate cancer. Lab Invest, 2008. **88**(12): p. 1340-8.

189. Willemarck, N., et al., Aberrant activation of fatty acid synthesis suppresses primary cilium formation and distorts tissue development. Cancer Res, 2010. **70**(22): p. 9453-62.

190. Schmidt, M.F. and M.J. Schlesinger, Relation of fatty acid attachment to the translation and maturation of vesicular stomatitis and Sindbis virus membrane glycoproteins. J Biol Chem, 1980. **255**(8): p. 3334-9.

191. Schlesinger, M.J., A.I. Magee, and M.F. Schmidt, Fatty acid acylation of proteins in cultured cells. J Biol Chem, 1980. **255**(21): p. 10021-4.

192. James, G. and E.N. Olson, Identification of a novel fatty acylated protein that partitions between the plasma membrane and cytosol and is deacylated in response to serum and growth factor stimulation. J Biol Chem, 1989. **264**(35): p. 20998-1006.

193. Olson, E.N. and G. Spizz, Fatty acylation of cellular proteins. Temporal and subcellular differences between palmitate and myristate acylation. J Biol Chem, 1986. **261**(5): p. 2458-66.

194. Magee, A.I. and S.A. Courtneidge, Two classes of fatty acid acylated proteins exist in eukaryotic cells. Embo J, 1985. **4**(5): p. 1137-44.

195. Triola, G., H. Waldmann, and C. Hedberg, Chemical biology of lipidated proteins. ACS Chem Biol, 2011. **7**(1): p. 87-99.

196. Resh, M.D., Trafficking and signaling by fatty-acylated and prenylated proteins. Nat Chem Biol, 2006. **2**(11): p. 584-90.

197. Webb, Y., L. Hermida-Matsumoto, and M.D. Resh, Inhibition of Protein Palmitoylation, Raft Localization, and T Cell Signaling by 2-Bromopalmitate and Polyunsaturated Fatty Acids. Journal of Biological Chemistry, 2000. **275**(1): p. 261-270.

198. Jennings, B.C., et al., 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. J Lipid Res, 2009. **50**(2): p. 233-42.

199. Hannoush, R.N. and J. Sun, The chemical toolbox for monitoring protein fatty acylation and prenylation. Nat Chem Biol, 2010. **6**(7): p. 498-506.

200. Resh, M., Use of analogs and inhibitors to study the functional significance of protein palmitoylation. Methods, 2006. **40**(2): p. 191-7.

201. Valdez-Taubas, J. and H. Pelham, Swf1-dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation. Embo J, 2005. **24**(14): p. 2524-32.

202. Resh, M.D., Targeting protein lipidation in disease. Trends Mol Med, 2012. **18**(4): p. 206-14.

203. Ducker, C.E., et al., Discovery and characterization of inhibitors of human palmitoyl acyltransferases. Mol Cancer Ther, 2006. **5**(7): p. 1647-59.

204. Politis, E.G., A.F. Roth, and N.G. Davis, Transmembrane topology of the protein palmitoyl transferase Akr1. J Biol Chem, 2005. **280**(11): p. 10156-63.

205. Roberts, P.J., et al., Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. J Biol Chem, 2008. **283**(37): p. 25150-63.

206. Drisdel, R.C., et al., Assays of protein palmitoylation. Methods

Protein Palmitoylation, 2006. **40**(2): p. 127-134.

207. Yang, Y.Y., J.M. Ascano, and H.C. Hang, Bioorthogonal chemical reporters for monitoring protein acetylation. J Am Chem Soc, 2010. **132**(11): p. 3640-1.

208. Yap, M.C., et al., Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry. J Lipid Res, 2010. **51**(6): p. 1566-80.

209. Wilson, J.P., et al., Proteomic analysis of fatty-acylated proteins in mammalian cells with chemical reporters reveals S-acylation of histone H3 variants. Mol Cell Proteomics, 2011. **10**(3): p. M110 001198.

210. Meiringer, C.T. and C. Ungermann, Probing protein palmitoylation at the yeast vacuole. Methods, 2006. **40**(2): p. 171-6.

211. Yang, W., et al., Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. Mol Cell Proteomics, 2010. **9**(1): p. 54-70.

212. Dowal, L., et al., Proteomic analysis of palmitoylated platelet proteins. Blood, 2011. **118**(13): p. e62-73.

213. Martin, B.R. and B.F. Cravatt, Large-scale profiling of protein palmitoylation in mammalian cells. Nat Methods, 2009. **6**(2): p. 135-8.

214. Kang, R., et al., Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. Nature, 2008. **456**(7224): p. 904-9.

215. Ivaldi, C., et al., Proteomic analysis of S-acylated proteins in human B cells reveals palmitoylation of the immune regulators CD20 and CD23. PLoS One, 2012. **7**(5): p. e37187.

216. Martin, B.R., et al., Global profiling of dynamic protein palmitoylation. Nat Methods, 2011. **9**(1): p. 84-9.

217. Wei, X., et al., Fatty acid synthase modulates intestinal barrier function through palmitoylation of mucin 2. Cell Host Microbe, 2012. **11**(2): p. 140-52.

218. Wei, X., et al., De novo lipogenesis maintains vascular homeostasis through endothelial nitric-oxide synthase (eNOS) palmitoylation. J Biol Chem, 2011. **286**(4): p. 2933-45.

219. Navarro-Lerida, I., et al., A palmitoylation switch mechanism regulates Rac1 function and membrane organization. Embo J, 2011. **31**(3): p. 534-51.

220. Omary, M.B. and I.S. Trowbridge, Covalent binding of fatty acid to the transferrin receptor in cultured human cells. J Biol Chem, 1981. **256**(10): p. 4715-8.

221. Alvarez, E., N. GironÃ¨s, and R.J. Davis, Inhibition of the receptor-mediated endocytosis of diferric transferrin is associated with the covalent modification of the transferrin receptor with palmitic acid. Journal of Biological Chemistry, 1990. **265**(27): p. 16644-16655.

222. Barker, P.A., et al., The low affinity neurotrophin receptor, p75LNTR, is palmitoylated by thioester formation through cysteine 279. J Biol Chem, 1994. **269**(48): p. 30645-50.

223. Shipston, M.J., Ion channel regulation by protein palmitoylation. J Biol Chem, 2011. **286**(11): p. 8709-16.

224. Hayashi, T., G.M. Thomas, and R.L. Huganir, Dual Palmitoylation of NR2 Subunits Regulates NMDA Receptor Trafficking. Neuron, 2009. **64**(2): p. 213-226.

225. Hayashi, T., G. Rumbaugh, and R.L. Huganir, Differential Regulation of AMPA Receptor Subunit Trafficking by Palmitoylation of Two Distinct Sites. Neuron, 2005. **47**(5): p. 709-723.

226. Gonnord, P., et al., Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. Faseb J, 2009. **23**(3): p. 795-805.

227. Kanaani, J., et al., A palmitoylation cycle dynamically regulates partitioning of the GABA-synthesizing enzyme GAD65 between ER-Golgi and post-Golgi membranes. J Cell Sci, 2008. **121**(Pt 4): p. 437-49.

228. Rathenberg, J., J.T. Kittler, and S.J. Moss, Palmitoylation regulates the clustering and cell surface stability of GABAA receptors. Mol Cell Neurosci, 2004. **26**(2): p. 251-7.

229. Amici, S.A., et al., A highly conserved cytoplasmic cysteine residue in the alpha4 nicotinic acetylcholine receptor is palmitoylated and regulates protein expression. J Biol Chem, 2012. **287**(27): p. 23119-27.

230. Foster, J.D. and R.A. Vaughan, Palmitoylation controls dopamine transporter kinetics, degradation, and protein kinase C-dependent regulation. J Biol Chem, 2011. **286**(7): p. 5175-86.

231. Delint-Ramirez, I., et al., Palmitoylation targets AKAP79 protein to lipid rafts and promotes its regulation of calcium-sensitive adenylyl cyclase type 8. J Biol Chem, 2011. **286**(38): p. 32962-75.

232. Zuckerman, D.M., et al., Differential regulation of two palmitoylation sites in the cytoplasmic tail of the beta1-adrenergic receptor. J Biol Chem, 2011. **286**(21): p. 19014-23.

233. Liu, R., et al., Palmitoylation regulates intracellular trafficking of beta2 adrenergic receptor/arrestin/phosphodiesterase 4D complexes in cardiomyocytes. PLoS One, 2012. **7**(8): p. e42658.

234. Tsutsumi, R., et al., Identification of G protein alpha subunit-palmitoylating enzyme. Mol Cell Biol, 2009. **29**(2): p. 435-47.

235. Adams, M.N., et al., The role of palmitoylation in signalling, cellular trafficking and plasma membrane localization of protease-activated receptor-2. PLoS One, 2011. **6**(11): p. e28018.

236. Claudinon, J., et al., Palmitoylation of interferon-alpha (IFN-alpha) receptor subunit IFNAR1 is required for the activation of Stat1 and Stat2 by IFN-alpha. J Biol Chem, 2009. **284**(36): p. 24328-40.

237. Zhao, Z., et al., Acyl-biotinyl exchange chemistry and mass spectrometry-based analysis of palmitoylation sites of in vitro palmitoylated rat brain tubulin. Protein J, 2010. **29**(8): p. 531-7.

238. Zambito, A.M. and J. Wolff, Palmitoylation of tubulin. Biochem Biophys Res Commun, 1997. **239**(3): p. 650-4.

239. Westermann, S. and K. Weber, Post-translational modifications regulate microtubule function. Nat Rev Mol Cell Biol, 2003. **4**(12): p. 938-47.

240. Lee, H., et al., Palmitoylation of caveolin-1 at a single site (Cys-156) controls its coupling to the c-Src tyrosine kinase: targeting of dually acylated molecules (GPI-linked,

transmembrane, or cytoplasmic) to caveolae effectively uncouples c-Src and caveolin-1 (TYR-14). J Biol Chem, 2001. **276**(37): p. 35150-8.

241. Kasahara, K., et al., Rapid trafficking of c-Src, a non-palmitoylated Src-family kinase, between the plasma membrane and late endosomes/lysosomes. Exp Cell Res, 2007. **313**(12): p. 2651-66.

242. Tanimura, N., et al., Palmitoylation of LAT contributes to its subcellular localization and stability. Biochem Biophys Res Commun, 2006. **341**(4): p. 1177-83.

243. Blanpain, C., et al., Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. J Biol Chem, 2001. **276**(26): p. 23795-804.

244. McCormick, P.J., et al., Palmitoylation controls recycling in lysosomal sorting and trafficking. Traffic, 2008. **9**(11): p. 1984-97.

245. Acconcia, F., et al., Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol. Mol Biol Cell, 2005. **16**(1): p. 231-7.

246. Li, L., M.P. Haynes, and J.R. Bender, Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4807-12.

247. La Rosa, P., et al., Palmitoylation regulates 17beta-estradiol-induced estrogen receptor-alpha degradation and transcriptional activity. Mol Endocrinol, 2012. **26**(5): p. 762-74.

248. Yang, X., et al., Palmitoylation of tetraspanin proteins: modulation of CD151 lateral interactions, subcellular distribution, and integrin-dependent cell morphology. Mol Biol Cell, 2002. **13**(3): p. 767-81.

249. Greaves, J. and L.H. Chamberlain, Differential palmitoylation regulates intracellular patterning of SNAP25. J Cell Sci, 2011. **124**(Pt 8): p. 1351-60.

250. He, Y. and M.E. Linder, Differential palmitoylation of the endosomal SNAREs syntaxin 7 and syntaxin 8. J Lipid Res, 2009. **50**(3): p. 398-404.

251. Lam, K.K., et al., Palmitoylation by the DHHC protein Pfa4 regulates the ER exit of Chs3. J Cell Biol, 2006. **174**(1): p. 19-25.

252. Baldwin, A.C., et al., A role for aberrant protein palmitoylation in FFA-induced ER stress and beta-cell death. Am J Physiol Endocrinol Metab, 2012. **302**(11): p. E1390-8.

253. Gao, X., et al., Membrane targeting of palmitoylated Wnt and Hedgehog revealed by chemical probes. FEBS Lett, 2011. **585**(15): p. 2501-6.

254. Hardy, R.Y. and M.D. Resh, Identification of N-terminal Residues of Sonic Hedgehog Important for Palmitoylation by Hedgehog Acyltransferase. J Biol Chem, 2012. **287**(51): p. 42881-9.

255. Abrami, L., S.H. Leppla, and F.G. van der Goot, Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. J. Cell Biol., 2006. **172**(2): p. 309-320.

256. Berzat, A.C., et al., Transforming activity of the Rho family GTPase, Wrch-1, a Wnt-regulated Cdc42 homolog, is dependent on a novel carboxyl-terminal palmitoylation motif. J Biol Chem, 2005. **280**(38): p. 33055-65.

257. Aittaleb, M., et al., Plasma membrane association of p63 Rho guanine nucleotide exchange factor (p63RhoGEF) is mediated by palmitoylation and is required for basal activity in cells. J Biol Chem, 2011. **286**(39): p. 34448-56.

258. Cheng, H., et al., S-palmitoylation of gamma-secretase subunits nicastrin and APH-1. J Biol Chem, 2009. **284**(3): p. 1373-84.

259. Stoeck, A., L. Shang, and P.J. Dempsey, Sequential and gamma-secretase-dependent processing of the betacellulin precursor generates a palmitoylated intracellular-domain fragment that inhibits cell growth. J Cell Sci, 2010. **123**(Pt 13): p. 2319-31.

Statement of the Problem

In the current state of clinical options, cancer-related mortality is primarily the result of a particular phenotype characterized as both invasive as well as resistant to most common therapeutic strategies. Many proteins have been linked to this phenotpye, which is stem-like and often attributable to an EMT, and the c-Met receptor seems to be one of the most common and potent linked thus far. A better understanding of the regulatory mechanisms controlling the expression of c-Met, and other similar proteins, could assist in the rational design of new therapeutic options to prevent or reverse this deadly phenotype. Effective treatment will require more than single modality approaches. It is necessary to identify broadly influential regulators of a particular disease profile in order to reduce the likelihood of resistance. The results described here will attest to the possibility that fatty acid synthesis and protein acylation are such globally influential targets. These two related processes have been shown to affect a multitude of proteins and may, in fact, drive the dysregulation of proteins in the diseased state. Our work directly connects them to the expression of c-Met which is characteristic of the lethal-phenotype in many cancers.

Chapter 1 introduces the initial studies that revealed a connection between FASN activity and c-Met protein expression. These findings were built upon in chapter 2 as it was determined that palmitate is covalently attached to c-Met and needed to maintain stability and transport of the receptor to the plasma membrane.

These results were specific to the c-Met receptor, but it may come to be found that other proteins similarly expressed in this phenotype are also responsive to acylation and lipogenesis. It is possible that these process work in conjunction to actually induce the transition to an

invasive and therapeutically-resistant cell population. Conversely, they may only play a passive, albeit necessary, role. Either determination would provide rationale for novel therapeutic strategies. Although cancer would be most obvious, the implications are not restricted to this disease but could impact any number of diseases associated with dysregulated protein localization due to acyl-group modifications. The work presented here represents a foundation upon which many more broad questions can be investigated.

CHAPTER 1

Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation

Reprinted from:
Coleman, D.T., Bigelow, B., and Cardelli, J. Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Mol. Cancer. Ther. 8(1): 214-224.

ABSTRACT

The HGF/c-Met signaling pathway is involved in the progression of a number of cancers and associated with increased tumor invasion and metastatic potential. We previously determined that the polyphenol epigallocatechin-3-gallate (EGCG) inhibited HGF-induced c-Met phosphorylation in a variety of tumor cell-lines, in part by disrupting lipid rafts. Fatty acid synthase (FASN) is implicated in cancer progression and may regulate lipid raft function. We therefore examined the effects of luteolin, a potent FASN inhibitor, on c-Met signaling. Luteolin blocked HGF-induced c-Met phosphorylation and scattering of DU145 prostate cancer cells. Western blot analysis indicated that inhibition of HGF-induced scattering by luteolin occurred coincident with reduction of total c-Met protein in DU145 cells. In addition, luteolin-induced c-Met downregulation was mimicked by a pharmacological inhibitor of FASN, C75, or FASN shRNA. Consistent with a role for FASN, loss of c-Met in cells treated with C75 or luteolin was prevented by exogenous addition of palmitate. Luteolin-induced loss of c-Met primarily occurred subsequent to transcription and translation, nor was it dependent on the activity of the 26S proteosome or acidic lysosomes. Taken together, our study demonstrates a novel connection between FASN activity and c-Met protein expression, and suggests that luteolin could act as a novel HGF/c-Met inhibitor by reducing expression of this receptor.

INTRODUCTION

Metastatic cancer is the primary cause of patient mortality, and therapeutic approaches to block this process are urgently needed. A major step of the malignant process is the transition of the stationary cancer cells to a motile mesenchymal-like phenotype [1] . This epithelial to mesenchymal transition is thought to be important in the loss of cell-cell adhesions and eventual invasion of the basement membrane — a prerequisite for metastasis [2]. A major contributor to the promotion of a mesenchymal phenotype is the HGF/c-Met signaling axis. Hepatocyte Growth Factor (HGF) is the only known ligand for the receptor tyrosine kinase (RTK) c-Met. Binding of HGF to c-Met leads to autophosphorylation of tyrosine residues within the cytoplasmic domain which function as docking sites for downstream effectors that mediate a number of cellular responses including proliferation, cell survival, actin remodeling, and motility [3, 4]. Prolonged induction of these pathways accounts for the cancer-promoting properties of the HGF/c-Met signaling axis. Overexpression of c-Met and activating mutations are seen in a number of cancers, including prostate and breast, and are strongly associated with aggressive disease [5-8].

Recently the interplay between distinct receptor tyrosine kinases has become better understood. For instance, c-Met overexpression leads to tumor cell resistance to EGFR-targeted therapy through heterodimerization that reestablishes downstream signaling [9]. Because of these unique RTK

partnerships, it has become clear that single target therapies will not be sufficient to inhibit tumor progression, and that multi-modality therapy, targeting several stimulatory pathways simultaneously, will prove more efficacious.

Phytochemicals, such as flavonoids, represent a source of relatively nontoxic, orally available and affordable compounds that are known to affect a number of different cancer-related pathways. Epidemiological studies have demonstrated a correlation between increased dietary intake of flavonoids with reduced risk of heart disease and cancer [10, 11]. Several anti-cancer properties attributed to these compounds include acting as anti-oxidants, the ability to interfere with various cancer promoting signaling pathways, and inhibition of growth factor receptors [11]. We and others have also found that EGCG appears to inhibit c-Met activation and EGFR activation by disrupting lipid rafts [12].

Lipid rafts are important plasma membrane regions which regulate cellular signaling in part through compartmentalization of growth factor receptors. We have demonstrated that the active form of c-Met resides in lipid rafts (Duhon *et al*., 2008 manuscript submitted), suggesting that disruption of lipid rafts may lead to inhibition of c-Met signaling and its downstream effects. FASN is the sole enzyme responsible for synthesis of long-chain saturated fatty acids and may play a role in regulating the activity of lipid rafts. In addition, many human cancers exhibit increased FASN expression correlating with advanced disease [13]. FASN activity has been proposed to maintain

membrane microdomain integrity and may promote cell survival involving a regulatory loop with the PI3K pathway [14, 15]. Brusselmann *et al.* ranked the inhibitory effects of a series of flavonoids on LNCap prostate cancer cell lipogenesis. These effects correlated with growth arrest, induction of apoptosis, reduced synthesis of phospholipids and cholesterol, and selective cytotoxicity of malignant cells. Of the compounds investigated by the Swinnen laboratory, the flavonoid luteolin was determined to have the greatest inhibitory effect on lipogenesis [16]. In addition, luteolin shares structural homology with known PI3K inhibitors, has been shown to inhibit FASN activity directly, and has strong antioxidant activity [16, 17].

Given the possible role of FASN activity in regulating lipid raft function, and the localization of active c-Met in lipid rafts, we investigated the effects of luteolin on the HGF/c-Met signaling axis. In this report we demonstrate that luteolin blocks HGF-induced scattering and motility of DU145 prostate cancer cells, and is a very potent inhibitor of the PI3K pathway. Moreover, we show that luteolin can downregulate c-Met expression through FASN inhibition, demonstrating a novel link between FASN activity and c-Met protein expression.

MATERIALS AND METHODS

Cell Culture

DU145, PC-3, H460, and MDA-MB-231 cell lines were obtained from ATCC and maintained in RPMI-1640 media, or DMEM/F-12 for 231s, (Cellgro, Herndon, VA, USA) containing 10% FBS (Gemini, CA, USA) and Penicillin/Streptomycin (Cellgro). Cells were maintained in a 37°C incubator with 5.0% CO_2.

Western Blot Analysis

Western blot analysis was performed as previously described [18]. Primary antibodies used were: phospho-Met (Y1234/1235), phospho-Met (Y1349), phospho-Met (Y1003), phospho-Akt (S473), phospho-Akt (S308), Akt, phospho-Erk, 4EBP1, phospho-JNK, FASN (Cell Signaling Technology, Beverly, MA, USA), phospho-S6K, total Erk, total c-Met (C-28) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and phospho-FAK (BD Transduction Laboratories, Franklin Lakes, NJ, USA), Actin (Sigma Aldrich, St. Louis, MO, USA), and Tubulin (Neomarkers, Fremont, CA, USA) were used as load controls. Blots were probed with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Pittsburgh, PA), and ECL (Amersham Biosciences) was used for protein detection. Recombinant Hepatocyte Growth Factor and LY294002 were obtained from EMB Biosciences (San Diego, CA, USA). Luteolin, C75, Apigenin, EGCG, Quercetin, Taxifolin, Lactocystin and Concanamycin A were obtained from Sigma Aldrich. MG132 was obtained

from Axxora. (San Diego, CA, USA). Palmitate (Sigma) was complexed to bovine serum albumin (Fischer) as described [16, 18, 19]. In short, palmitate was dissolved in ethanol to 150 mM and 1 volume was added to 4 volumes of a 4% bovine serum albumin solution in .9% NaCl and incubated for 1 hr at 37% for a 30 mM stock of BSA-complexed palmitate.

RT-PCR

DU145 cells were plated and allowed to grow to confluency on a 10 cm dish in serum-containing media. Cells were incubated ± 25 μM luteolin for 8 hours. Cells were homogenized in Trizol (Invitrogen Corp., Carlsbad, CA, USA), and total RNA was isolated according to manufacturer's protocol. RNA was subjected to reverse-transcriptase PCR for a range of cycles. The primer set for c-Met were purchased from Integrated DNA Technologies (Coralville, IA, USA): Forward 5' – AGG CAC TAG CAA AGT CCG AGA TGA – 3', Reverse 5' – GGA AAC AAT CTG GGT GTT CCA GCA – 3'. PCR products were run on a 1% agarose gel. GAPDH PCR products were used as a loading control.

Immunofluorescence Microscopy

DU145 were grown on 4-well plastic slides (NalgeNunc, Rochester, NY, USA) in serum-containing media. Following treatment, cells were fixed in 4% PFA for 20 min and incubated for 1 hour at room temperature with primary tubulin, phosphotyrosine (Cell Signaling), or c-Met (R&D Systems, MN, USA)

antibody suspended in BSP (Bovine Serum Albumin, Saponin, PBS). Wells were washed with PBS and Texas Red-dye conjugated secondary antibody was applied suspended in BSP along with phalloidin. Wells were washed again and Antifade/DAPI (Invitrogen) was added prior to setting coverslips. Fluorescent images were taken using an Olympus BX-50 epifluorescence microscope using MetaMorph software.

RNAi

DU145 cells stably expressing shRNAs targeting FASN or non-target shRNA were generated using Mission Lentiviral Transduction Particles from Sigma-Aldrich. Five separate cell lines were established using five lentivirus clones according to the manufacturer's protocol.

Scattering and Motility Assays

For the scattering assay, $4x10^4$ DU145 cells, plated on a 24-well plate and grown in serum-containing media, were pretreated with the indicated concentrations of luteolin for the appropriate time. HGF was then spiked into wells to give a final concentration of 33 ng/ml and incubated overnight. For the motility assay, $4.5x10^5$ DU145 cells were plated on a 6-well plate to form a confluent monolayer in serum-containing media. Cells were pretreated for 1 hr ± 25 μM luteolin. Intersecting scratches were made across the plate with a pipette tip and washed with PBS to remove cell debris. Serum-free media ± 15 μM luteolin ± 33 ng/ml HGF was then added to the cells and incubated

overnight. The scratch for a representative T_0 was made immediately prior to fixing. For each experiment, cells were fixed with PFA and stained with phalloidin, and representative images were taken using an Eclipse TE300 inverted microscope (Nikon, Japan).

RESULTS

Pretreatment with Luteolin Prevents HGF-Induced Scattering and Motility of Prostate Cancer Cells.

We have demonstrated that the addition of the flavonoid EGCG blocks the HGF-induced signaling and scattering of DU145 tumor cells, via disruption of lipid rafts (Duhon *et al.,* 2008; manuscript submitted). To determine if the flavonoid luteolin, a known FASN inhibitor, also affected the HGF-induced phenotypic change, DU145 cells were pretreated overnight with a range of luteolin concentrations prior to stimulation with HGF (33 ng/ml) for 18 hours. Luteolin blocked HGF-induced scattering in a dose-dependent fashion, and this inhibition of scattering was more pronounced with overnight pretreatment compared to one hour pretreatment (Fig. 1A). To address this pretreatment time-dependent effect, we pretreated cells with 25 μM luteolin for varying periods of time from 1-12 hours prior to HGF addition. An increase in inhibition of HGF-induced scattering was observed beginning at 4 hours of luteolin pretreatment with a greater percentage of cells remaining adherent in colonies with 8 hours or more of pretreatment time (Fig. 1B).

In addition to blocking HGF-induced scattering, luteolin caused a change in cell morphology (Fig. 1; compare panel Ai with Av). Cells appeared flatter and larger in diameter (Fig. 2A). To more closely examine the effects of luteolin on cell morphology, we performed immunofluorescence (I.F.) microscopy on DU145 cells treated with 25 μM luteolin for 9 hours. Cells

were fixed and stained to visualize actin and tubulin. Figure 2A demonstrates that luteolin caused a reduction in the number of F-actin stress fibers, but had no apparent effect on microtubule distribution.

Actin stress fiber formation is an important contributor to cell motility and accordingly we performed a wound-healing motility assay to determine the effects of luteolin on HGF-induced motility and wound closure. Confluent monolayers of DU145 cells were incubated with or without 25 μM luteolin for one hour, after which a scratch was made with a pipette tip to wound the monolayer. The media was aspirated and fresh serum-free media was added with or without 15 μM luteolin and HGF at 33 ng/ml. Serum-free media was used so that wound closure due to motility would not be obscured by closure due to normal cell growth and proliferation. A T_0 scratch was also made immediately prior to fixing the cells in paraformaldehyde (Fig. 2Bi). The control cells, not treated with HGF, failed to migrate into the cleared area (Fig. 2Bii), while the HGF-stimulated cells almost entirely closed the wound area (Fig. 2Biii). Luteolin, however, blocked the HGF-stimulated wound-closure (Fig. 2Biv), suggesting that luteolin blocks HGF-mediated cell motility (Fig. 2B). Trypan blue staining revealed that luteolin had no affect on cell viability (results not shown).

Cell attachment to the substratum is mediated by focal adhesions, and dynamic focal adhesion turnover is essential for cell motility [20]. To determine if luteolin inhibits cell migration by lowering the number of focal adhesion sites, DU145 cells were treated with 25 μM luteolin for 9 hours, and

actin and phosphotyrosine residues were visualized by I.F. microscopy. Focal adhesions were identified as areas densely populated with phosphorylated proteins localized at the tips of actin fibers. As shown in Figure 2C, there was no difference in the size or quantity of focal adhesions between control and luteolin treated cells, suggesting that luteolin does not affect focal adhesion formation or stability.

Luteolin Pretreatment is required to Attenuate c-Met Activation by HGF, but Pretreatment is not required for Inhibition of Akt-phosphorylation

To determine if luteolin affected HGF/c-Met signaling pathways, thus blocking cell scattering and motility, we treated DU145 cells with 25 μM luteolin for 1, 4, 8, or 12 hours prior to HGF treatment for 20 minutes. As shown in Figure 3A, Western blot analysis of whole cell lysates using antibodies specific for c-Met phosphorylation showed that HGF-induced c-Met phosphorylation at tyrosine residues 1234/1235 (the kinase domain) and 1349 (an adaptor docking site) was significantly attenuated, but only after pretreatment with luteolin for greater than 1 hour (Fig. 3A); moreover attenuation increased with pretreatment time. However, luteolin blocked phosphorylation of Akt at two separate residues within one hour of pretreatment. Additional experiments demonstrated luteolin blocked phosphorylation of Akt when pretreated for only 10 minutes before the addition of HGF (results not shown). Conversely, HGF-mediated activation of two

MAP kinases, JNK and Erk, along with focal adhesion kinase (FAK) was not affected by luteolin pretreatment (Fig. 3A).

Since Akt phosphorylation, indicative of PI3K activity, was sensitive to short preincubation times with luteolin, we further investigated the role of luteolin in HGF-dependent PI3K activity. To determine if luteolin affected the activity of PTEN, a phosphatase that negatively regulates PI3K signaling, a dose response experiment was performed using DU145 cells and the PTEN -/- prostate cancer cell line PC-3 [21, 22]. Western blot analysis indicated that luteolin inhibited phosphorylation of Akt similarly in both cell lines with an IC_{50} of approximately 5 μM, suggesting that luteolin acts directly as a PI3K inhibitor rather than by activating PTEN (Figures 3B and C). Since luteolin pretreatment caused an apparent reduction of PI3K activity within 1 hour, followed by inhibition of c-Met phosphorylation beginning at 1 hour, we tested if the loss of PI3K activity negatively regulated c-Met phosphorylation. To test this hypothesis, we used the PI3K inhibitor, LY294002, to determine if prolonged inhibition of PI3K resulted in the reduction of HGF-induced c-Met phosphorylation. As shown in Figure 3D, LY294002 pre-treatment over a time course similar to luteolin pre-treatment did not affect c-Met phosphorylation; despite inhibiting HGF-induced Akt phosphorylation. Therefore, the ability of luteolin to attenuate c-Met phosphorylation is independent of its ability to inhibit PI-3K.

Luteolin Pretreatment Induces a Post-Transcriptional Loss of c-Met Most Likely Through Inhibition of FASN

To further investigate the mechanism of luteolin-mediated decrease in HGF-mediated c-Met activity, we performed Western blot analysis using whole cell lysates from DU145 cells treated with luteolin for various times. Figure 4A shows that luteolin caused a reduction in the level of total c-Met protein beginning as early as 1 hour, leading to a greater than 90 percent loss by 12 hours. The loss of c-Met expression over time mirrors the loss of HGF-induced c-Met phosphorylation seen in Figure 3A, suggesting that the mechanism of luteolin-mediated downregulation of c-Met activity may be primarily through decreased receptor expression. In order to confirm the generality of these findings, we treated several additional cancer cell lines, including the prostate tumor cell-line PC-3, the breast cancer cell line MDA-MB-231, and the lung cancer cell line H460, with 25 μM luteolin for 9 hours. Luteolin treatment reduced c-Met expression in all cell lines (Fig. 4A, right panel), suggesting that the observed effects of luteolin on c-Met receptor levels are not cell context dependent.

Since luteolin is a known FASN inhibitor, we predicted that a specific FASN inhibitor, C75, might also induce a loss of c-Met expression [23]. As seen in Figure 4B, the addition of C75 resulted in the loss of c-Met with kinetics similar to that observed for luteolin treatment. To more directly determine the role of FASN in controlling c-Met levels, we expressed FASN-specific shRNA via lentivirus to generate stable cell-lines. As demonstrated in

Figure 4C, the FASN shRNA cell-lines contained greatly reduced FASN levels, and this was paralled by comparable decreases in c-Met levels. Finally, the addition of palmitate, the end product of FASN catalytic activity, prevented luteolin- (Fig. 4D, left panel) and C75-induced (right panel) c-Met loss in a dose-dependent manner. These experiments suggest a potential novel link between FASN activity and c-Met expression levels.

To determine if luteolin affected c-Met transcription, thus accounting for loss of c-Met protein, we performed reverse-transcriptase PCR on RNA prepared from DU145 cells cultured alone or in the presence of luteolin for 8 hours. Gel electrophoresis of PCR products at different cycle times indicated that luteolin treatment only reduced c-Met mRNA by approximately 20 percent in DU145 cells relative to GAPDH RNA controls (Fig. 5A); real-time PCR confirmed this 20 percent reduction (data not shown). The modest loss of c-Met mRNA compared to the greater than 90 percent loss of c-Met suggests a post-transcriptional level of control is involved. The PI3K/Akt pathway regulates cap-dependent translation in part by affecting 4EBP1 through mTOR activation, leading to eIF4E availability to initiate translation of mRNAs with complex 5' UTRs [24]. The c-Met mRNA is predicted to have a 5' UTR that might be eIF4E responsive (unpublished results). Accordingly, we used rapamycin, a specific mTOR inhibitor, and LY294002 to inhibit the PI3K/Akt/mTOR pathway. Western blot analysis revealed that inhibition of this pathway, as indicated by the blocked phosphorylation of downstream S6 kinase and the shift of 4EBP1 to a hypo-phosphorylated form in cells exposed

to HGF, did not result in a decrease in c-Met levels (Fig. 5B). This suggests that in DU145 cells c-Met mRNA translation is most likely not dependent on the mTOR pathway and that luteolin is not lowering c-Met protein levels through inhibition of cap-dependent translation.

To further address the possible mechanism of action accounting for luteolin-induced loss of c-Met, we compared the rate of c-Met loss upon treatment with the eukaryotic protein synthesis inhibitor, cycloheximide, alone or in combination with luteolin. Western blot analysis indicated an increased rate of c-Met loss in the presence of cycloheximide and luteolin as compared to treatment with either agent alone (data not shown). If luteolin was inhibiting c-Met protein synthesis like cycloheximide, we would not observe this increased rate of c-Met loss when combined with cycloheximide. Increased doses of cycloheximide did not increase the rate of c-Met loss suggesting the dose used was optimal (data not shown). Together, these data suggest luteolin promotes c-Met degradation of already synthesized c-Met protein.

Luteolin-induced Loss of c-Met is Independent of the Proteosomal and Lysosomal Degradation Pathways

When the c-Met receptor is activated by HGF, the juxtamembrane tyrosine residue 1003 is phosphorylated, which recruits the ubiquitin ligase Cbl. Ubiquitination prompts internalization of the receptor and trafficking for degradation via either the 26S proteosome or by trafficking to acidic lysosomes [25-29]. Western blot analysis indicated that HGF treatment of DU145 cells

resulted in loss of c-Met protein within 2 hours, preceded by phosphorylation at Y1003 (Fig. 6A, 6B). In contrast, luteolin treatment did not result in phosphorylation at Y1003 (Fig. 6B). We used specific pharmacological inhibitors to determine if luteolin-induced c-Met loss was dependent on the proteosomal or the lysosomal degradation pathway. MG132 and lactacystin are inhibitors of the 26S proteosome, and concanamycin A inhibits acidification of lysosomes, reducing proteolysis. DU145 cells were pretreated with the indicated inhibitors prior to HGF or luteolin treatment and probed for c-Met loss by Western blot analysis. HGF treatment resulted in a rapid loss of c-Met, which was partially prevented by inhibition of the 26S proteosome or by blocking lysosomal acidification (Fig. 6C). In contrast, luteolin-induced c-Met loss was not prevented in the presence of these inhibitors (Fig. 6C). In addition, inhibiting both the 26S proteosome and lysosomal acidification in combination prevented HGF-induced degradation (Fig. 6C bottom panel), but again luteolin-induced c-Met downregulation was not blocked by the combination of inhibitors (Fig. 6C bottom panel). These experiments suggest the proteosomal and lysosomal pathways do not play a major role in luteolin-induced c-Met loss, and suggest a potentially novel mechanism of action to account for the effects of this flavonoid on c-Met levels.

To confirm that a reduction in c-Met levels is the result of loss of c-Met from the cell surface, cells were treated with HGF or luteolin for the times indicated in Figure 6D. I.F. microscopy using a c-Met antibody indicated that HGF treatment rapidly resulted in redistribution of c-Met from the cell surface

to vesicles residing near the nucleus. Cell surface c-Met was also reduced by luteolin treatment, but the intracellular distribution was less vesicular, although c-Met did accumulate near the nucleus during the time period levels were decreasing.

The 2,3 Double Bond in Ring C of Flavonoids is Important for c-Met Down Regulation

In order to determine if structurally related flavonoids have similar effects on c-Met levels when compared to luteolin, we treated DU145 cells for 9 hours with the flavones luteolin and apigenin, the flavonol quercetin, the flavanone taxifolin, and the flavanol EGCG. Western blot analysis indicated that luteolin, apigenin, and quercetin all reduced c-Met expression to similar levels, but that taxifolin and EGCG had no significant effect on c-Met expression at these concentrations (Supplemental Figure 1A). Interestingly, each compound effective at lowering c-Met levels (luteolin, apigenin, and quercetin) contained a double bond between carbons 2 and 3 of the center six-member oxygen-containing C ring. Comparatively, taxifolin, ineffective at lowering c-Met expression, lacks this double bond which is the only difference from quercetin's molecular structure (Supplemental Figure 1B). These data reveal that structurally-similar flavonoids have similar effects on c-Met expression, and that the 2, 3 double bond of the C-ring is important for this effect.

DISCUSSION

Some phytochemicals, such as EGCG from green tea extract, have potent inhibitory activity against growth factor signaling pathways, including HGF/c-Met [18, 30]. In this report, we investigated the effects of the flavonoid luteolin on the HGF/c-Met signaling axis, and found that it blocks c-Met signaling through a mechanism unique from EGCG. Our data suggest that one key mechanism of action is through inhibition of FASN, leading to a post-transcriptional reduction in c-Met protein levels.

In the presence of HGF, DU145 prostate cancer cells lose cell-cell adhesions and acquire a motile phenotype. EGCG blocks HGF-induced scattering with no preincubation time required [18]; however, we report here that luteolin had a minimal effect added with HGF, and that a preincubation time was required to block c-Met phosphorylation. This result suggests that the two compounds are working by different mechanisms. Also, luteolin caused changes in cell morphology (reduction in stress fibers and cell flattening) not observed with EGCG treatment. Similar effects by luteolin on the actin cytoskeleton were shown by Lee *et al.* in the context of HepG2 hepatoma cells [31]. How luteolin effects these changes remains to be determined, but we speculate that luteolin disrupts actin-remodeling proteins, such as the Rho family of small GTPases and downstream effectors like Rho-kinase (ROCK). This hypothesis is supported by Hendricks *et al.* who have previously shown that luteolin can inhibit RhoA activity in a monocyte system [32]. Actin stress

fibers are important for cell motility, and disruption of actin stress fibers caused by luteolin treatment may account for the compound's ability to block HGF-induced scattering and motility, although this remains to be tested [33].

Luteolin also rapidly blocked HGF-induced Akt phosphorylation, while 1hour of pretreatment was required to inhibit c-Met phosphorylation. We conclude luteolin is working directly as a PI3K inhibitor, rather than blocking ligand-induced receptor activation. Consistent with this possibility, others have found that luteolin can inhibit PI3K in an *in vitro* assay [34]. Furthermore, luteolin was equally effective at blocking Akt phosphorylation in a PTEN -/- prostate cancer cell line; thereby ruling out the possibility that luteolin activated this phosphatase to negatively regulate PI3K activity. Loss or inactivation of PTEN is common in a number of cancers including prostate cancer, and aberrant signaling through the PI3K pathway has been implicated in tumor cells becoming resistant to receptor tyrosine kinase-targeted therapies [21, 22]. Therefore, the ability of luteolin to target PI3K signaling independent of PTEN status emphasizes the potential therapeutic activity of this phytochemical.

Furthermore, the time-dependent attenuation of HGF-mediated c-Met phosphorylation by luteolin was independent of the effects on Akt phosphorylation, since prolonged treatment of cells with LY294002 did not block the ability of HGF to induce c-Met phosphorylation. Activation of the Erk and JNK signaling pathways were not affected by luteolin even after long preincubation when c-Met was no longer maximally activated by HGF. This

suggests that sufficient active c-Met remained to induce phosphorylation of Erk and JNK. FAK was constitutively phosphorylated at high levels in DU145 cells, and phosphorylation was only minimally attenuated with prolonged luteolin preincubation.

Our results are consistent with Lee *et al.* who have shown that luteolin blocked HGF-induced Akt phosphorylation, but only partially affected MAP kinases in hepatoma cells [31]. In addition, however, our results show a requirement for pretreatment greater than 1 hour to affect c-Met phosphorylation, whereas only a short pretreatment was required to block phosphorylation of Akt. We speculate that the requirement for long pretreatment could reflect the length of time required to alter the concentration of a factor(s) regulating c-Met. One of these factors could be levels of palmitate that regulates lipid raft activity or integrity. One can rule-out a delay in the compound crossing the cell membrane, because of the rapid effects on PI3K activity.

Interestingly, the reduction of HGF-induced phosphorylated of c-Met was not the result of luteolin blocking ligand-induced activation of the receptor, but instead appeared to be due to luteolin-mediated downregulation of total c-Met protein. RT-PCR analysis demonstrated no dramatic change in total c-Met mRNA levels, suggesting luteolin was acting primarily at a post-transcriptional level. This result was observed in multiple tumor cell-lines. Similar to our results, it has been reported that apigenin and luteolin downregulate HER2 expression in HER2/neu-overexpressing breast cancer cell

lines, although the mechanism regulating this was not defined for luteolin [35]. A subsequent report suggested that luteolin induced loss of HER2 at a post-transcriptional level [36]. We have observed similar effects of luteolin and apigenin on HER2 expression in DU145 prostate cancer cells (unpublished data).

C75, a specific pharmacological inhibitor to FASN, as well as FASN-specific shRNA reduced the level of total c-Met protein in DU145 cells. FASN is the sole enzyme responsible for *de novo* synthesis of long-chain unsaturated fatty acids, primarily the 16 carbon fatty acid palmitate. The addition of exogenous palmitate to the system prevented the C75- and luteolin- induced loss of c-Met, further supporting a role or FASN in maintaining c-Met expression levels. Malignant cells have a much greater reliance on *de novo* synthesized fatty acids as opposed to exogenously-derived fatty acids, suggesting that FASN could be a good therapeutic target [37]. The need of malignant cells for high expression of FASN has been attributed to maintenance of the lipid supply required by highly proliferative cells, regulation of stimulatory signaling pathways through palmitoylation of proteins and stabilizing membrane domains, as well as restoration of oxidation potential through consumption of NADPH under hypoxic conditions [13]. A number of cellular receptors, including c-Met, require localization within ordered lipid microdomains (lipid rafts) for efficient signaling [38, 39]. Lipid rafts are rich in cholesterol and sphingolipids, products generated in tumors cells by FASN

[15, 40]. Our results suggest that higher FASN activity maintains lipid rafts which may help stabilize levels of c-Met.

Our results suggest that, in DU145 cells, translation of c-Met mRNA was not regulated by the PI3K/Akt/mTOR/eIF4E pathway, and therefore inhibition of PI3K is not the mechanism responsible for luteolin-induced c-Met loss. Our data also suggest that the loss of c-Met induced by luteolin occurred in part subsequent to c-Met synthesis, but was independent of an active proteosome or acidic lysosomes.

The c-Met receptor, similar to other RTKs, has been reported to undergo a mechanism of ligand-induced negative regulation through internalization and degradation [26, 28, 29]. Using inhibitors to both proteosomal activity and acidification of lysosomes, we show that neither of these pathways was significantly involved in luteolin-induced c-Met loss. In contrast, Chiang *et al.* concluded that luteolin induced loss of HER2 in breast carcinoma cells was blocked by the addition of proteosomal inhibitors [36].

Another mechanism of c-Met downregulation is receptor shedding, which is mediated by metalloproteinases that cleave the extracellular domain of the receptor promoting degradation of the cytoplasmic domain [41]. Our results speak against this possibility for three reasons. Firstly, no c-Met protein was detected in the media following luteolin treatment (data not shown). Secondly, similar trends of c-Met loss were detected with antibodies to both a cytoplasmic domain and an extracellular domain (data not shown). Finally, I.F.

microscopy indicated that reduction of c-Met levels was accompanied by internalization of the receptor into intracellular compartments.

An additional possibility for c-Met loss is that luteolin is stimulating an apoptotic response resulting in activation of caspases that subsequently degrade the receptor. However, no significant apoptosis was observed in treated cells during these time points by microscopy or protein analysis (data not shown). We conclude that luteolin is acting through a novel mechanism of receptor degradation unique from that induced by ligand or other known stimuli; although the mechanism remains to be defined.

We determined, using a series of structurally similar compounds, that the 2,3 double bond of the C ring of luteolin conveys the activity required for downregulation of c-Met. We show that the flavones luteolin and apigenin along with the flavonol quercetin, each containing the 2-3 double bond and having the most potent inhibitory activity toward FASN, as shown by Swinnen *et al*., have the greatest effect on c-Met levels [16]. Apigenin has previously been reported to downregulate HER2 by proteosomal degradation in breast cancer cells [42]. We hypothesize that by targeting FASN these compounds can downregulate the expression of a number of cancer-associated growth factor receptors that require membrane microdomains stabilized by FASN activity.

Our study provides evidence for a potential link between FASN activity controlling levels of c-Met perhaps by stabilization, and that luteolin could be a potential therapeutic agent to down regulate c-Met levels through inhibition of

FASN. Overexpression of FASN has been reported to increase activity of the HER1 and HER2 receptors in breast cancer cells and may be important in resistance to Trastuzumab [43, 44]. We hypothesize that increased expression of FASN could stabilize growth factor receptors localized in lipid rafts, such as c-Met, thereby promoting cancer progression. Our results, consistent with other published studies, demonstrate that luteolin inhibits PI3K activity in prostate cancer cells. FASN expression is, in part, controlled by a regulation loop with PI3K, and is inversely correlated with PTEN expression [14, 45]. Consequently, luteolin has the potential to target both PI3K and FASN activities to disrupt growth factor receptor stability and cancer progression, suggesting it might be more effective than either PI3K inhibitors or FASN inhibitors given alone.

ACKNOWLEDGMENTS

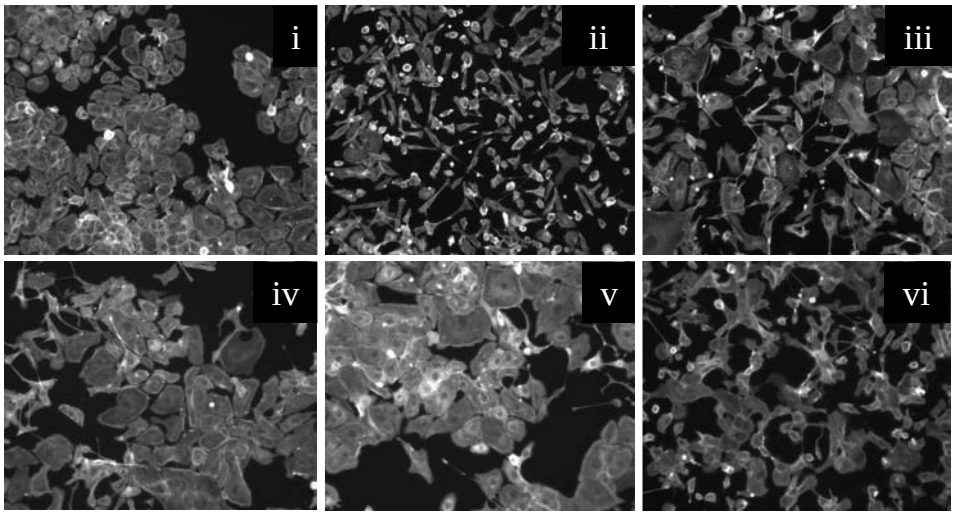
The authors would like to thank Dr. Jared Snider and Josh Steffan for their critical reading of the manuscript. This work was supported in part by a grant from the National Institutes of Health (NIH R01 CA104242-01).

CHAPTER 1 FIGURE LEGEND

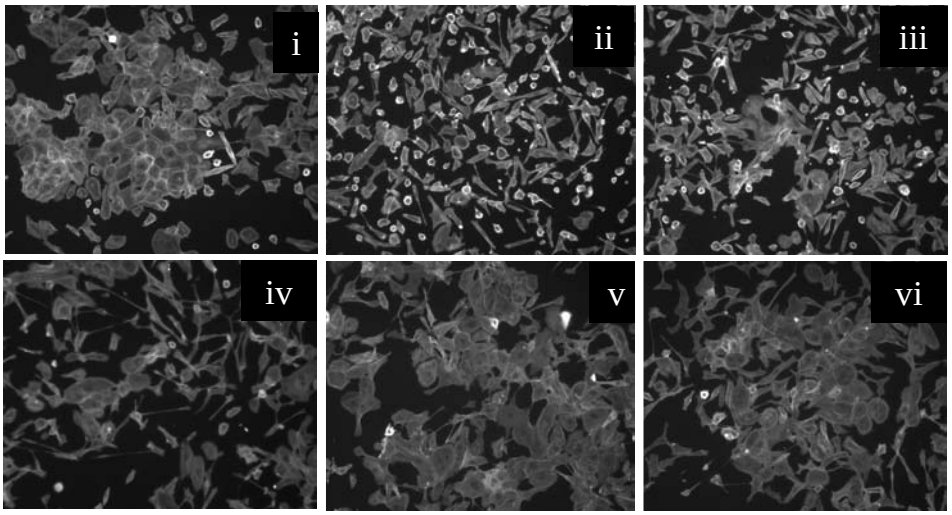
Fig. 1. Luteolin pretreatment blocks HGF-induced cell scattering. (A) DU145 prostate cancer cells were pretreated with DMSO (i and ii) or increasing luteolin concentrations (iii—10 μM; iv—15 μM; v and vi —30 μM) for one hour (vi) or overnight (iii-v) prior to HGF stimulation (ii-vi—33 ng/mL) for 18 hours. **(B)** DU145 cells were incubated with DMSO (i and ii) or 25 μM luteolin for 1 hour (iii), 4 hours (iv), 8 hours (v), or 12 hours (vi) prior to HGS stimulation (ii-vi). Cells were fixed, stained for actin, and representative images captured.

CHAPTER 1 FIGURE

A.



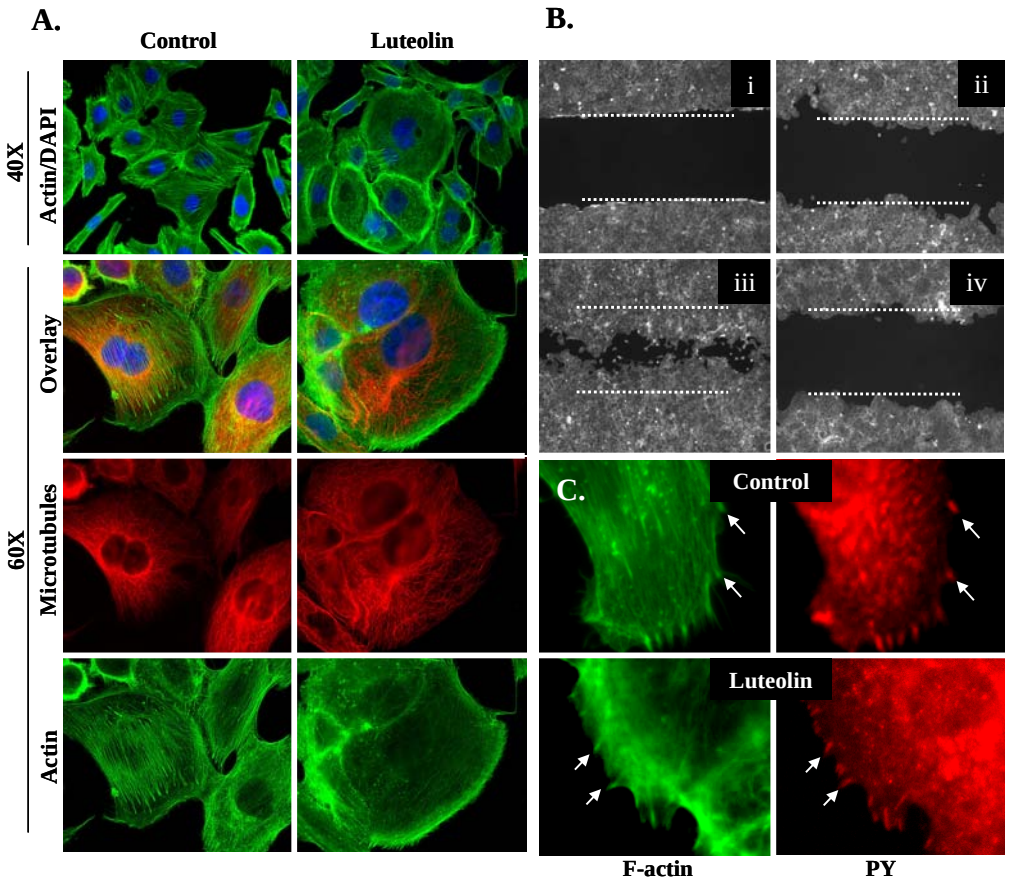
B.



CHAPTER 1 FIGURE LEGEND

Fig. 2. Luteolin disrupts actin stress fibers and blocks HGF-induced cell motility, but does not affect focal adhesions. (A) DU145 cells were treated with DMSO or 25 μM luteolin for 12 hours. Cells were fixed, stained for actin, DAPI, and microtubules, and representative 40X and 60X images were captured. **(B)** Confluent DU145 monolayers were pretreated with DMSO (ii and iii) or 25μM luteolin (iv) for 1 hour. The monolayer was wounded, washed, and fresh serum-free media containing 15 μM luteolin (iv) or DMSO (ii and iii) was added. Cells were then incubated alone (ii) or with HGF (iii and iv—33 ng/mL) overnight. The following day, one monolayer was wounded as an untreated control (i), after which all cells were fixed and stained for actin. **(C)** DU145 cells were incubated with DMSO or 25μM luteolin for 12 hours. Cells were fixed, stained for actin or phosphotyrosine, and representative images were captured. Focal adhesions, represented by areas of actin and phosphotyrosine colocalization, are indicated by arrows.

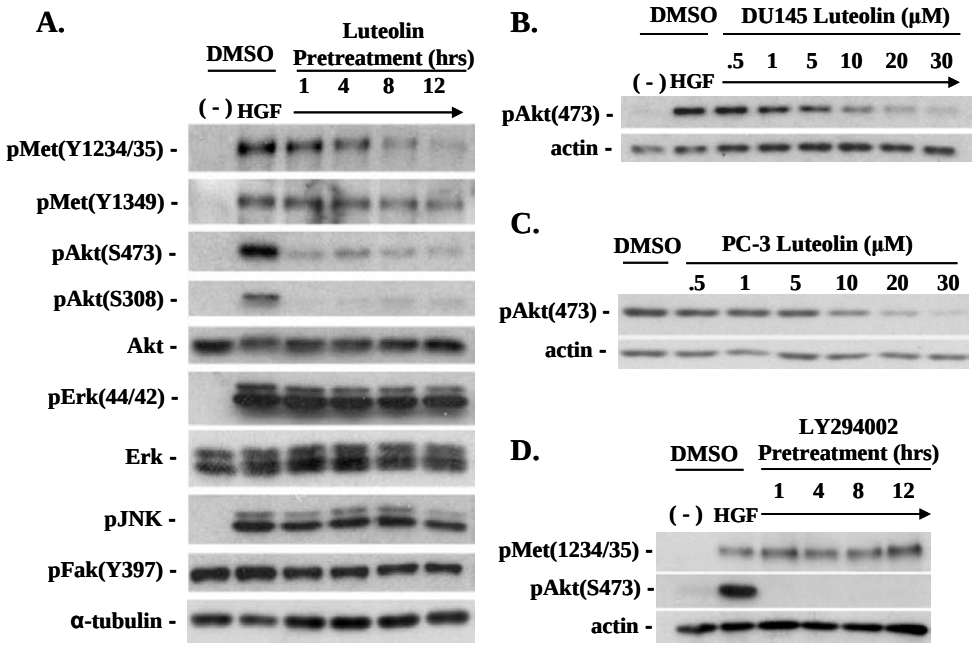
CHAPTER 1 FIGURE



CHAPTER 1 FIGURE LEGEND

Fig. 3. Luteolin inhibits PI3K and blocks HGF-induced c-Met phosphorylation with prolonged pretreatment. (A) DU145 cells were pretreated with DMSO or 25 μM luteolin for 1, 4, 8, or 12 hours prior to HGF (33ng/ml) stimulation for 20 minutes. (**B)** DU145 cells were pretreated with DMSO or indicated luteolin concentrations for 1 hour prior to HGF treatment for 20 minutes. **(C)** PC-3 prostate cancer cells were treated with DMSO or indicated concentrations of luteolin for 1 hour. **(D)** DU145 cells were pretreated with DMSO or 20 μM LY294002 for 1, 4, 8, or 12 hours prior to HGF stimulation. For each experiment, whole cell lysates were collected and probed by western blot analysis using indicated antibodies. Actin or tubulin was also probed as a load control.

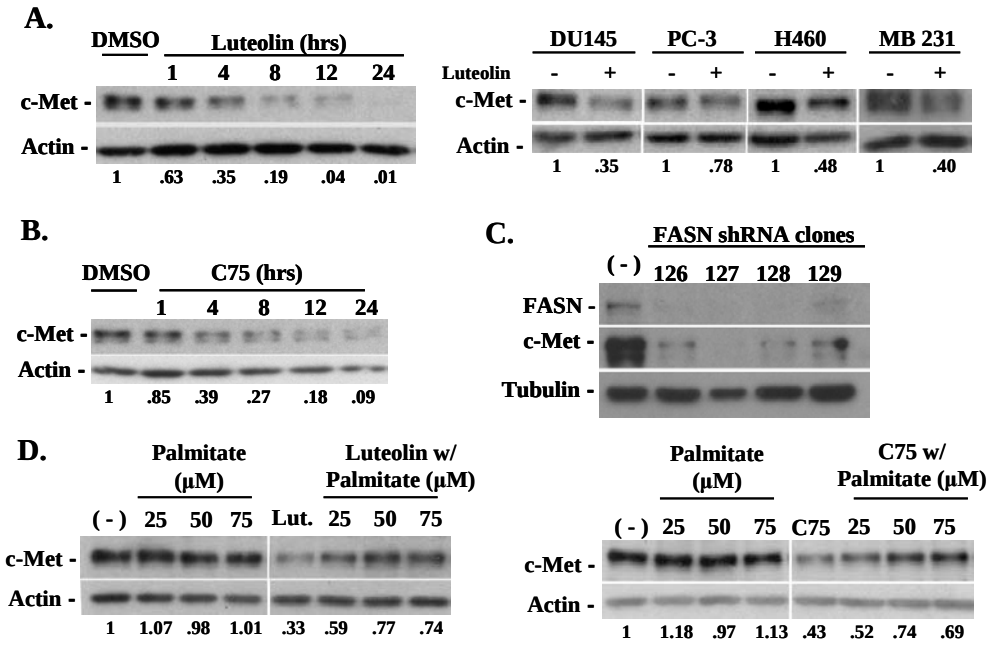
CHAPTER 1 FIGURE



CHAPTER 1 FIGURE LEGEND

Fig. 4. **Luteolin reduces c-Met levels through inhibition of fatty acid synthase. (A, left panel)** DU145 cells were treated with DMSO or 25 μM luteolin for 1, 4, 8, 12, or 24 hours. (**A, right panel)** DU145, PC-3, the lung cancer cell line H460, and breast cancer cell line MDA-MB-231 were treated for 9 hours with DMSO (-) or 25 μM luteolin (+). **(B)** DU145 cells were treated with DMSO or 25 μM C75 for 1, 4, 8, 12, or 24 hours. **(C)** DU145 cells were stably transduced with lentivirus expressing non-target shRNA (-) or one of four FASN-targeted shRNA clones (126,127, 128, or 129). **(D, left panel)** DU145 cells were pretreated with DMSO or 25 μM luteolin **(right panel)** or 25 μM C75 prior to incubation with palmitate-BSA for 9 hours. For each experiment, whole cell lysates were collected and probed by western blot analysis using c-Met or FASN-specific antibodies. Actin was also probed as a load control. Densitometry was performed on appropriate blots and shown as fold change of control.

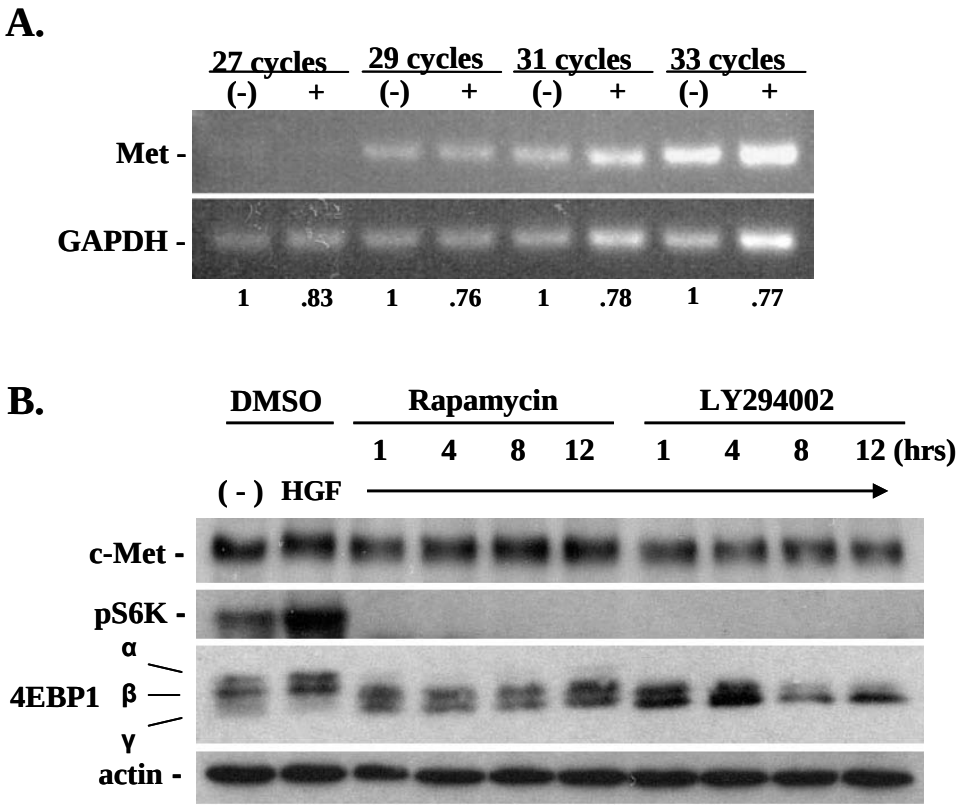
CHAPTER 1 FIGURE



CHAPTER 1 FIGURE LEGEND

Fig. 5. Luteolin post-translationally regulates c-Met levels. (A) DU145 cells were treated with DMSO (-) or 25 uM luteolin (+) for 8 hours. RNA was then isolated and RT-PCR was performed using primers for c-Met, and for GAPDH as control at different cycles. (**B)** DU145 cells were pretreated with DMSO, rapamycin (100 ng/ml), or LY294002 (20 μM) for indicated time periods prior to HGF stimulation (33ng/ml) for 20 minutes. Lysates were collected and probed by western blot analysis using indicated antibodies. Actin was used as a load control.

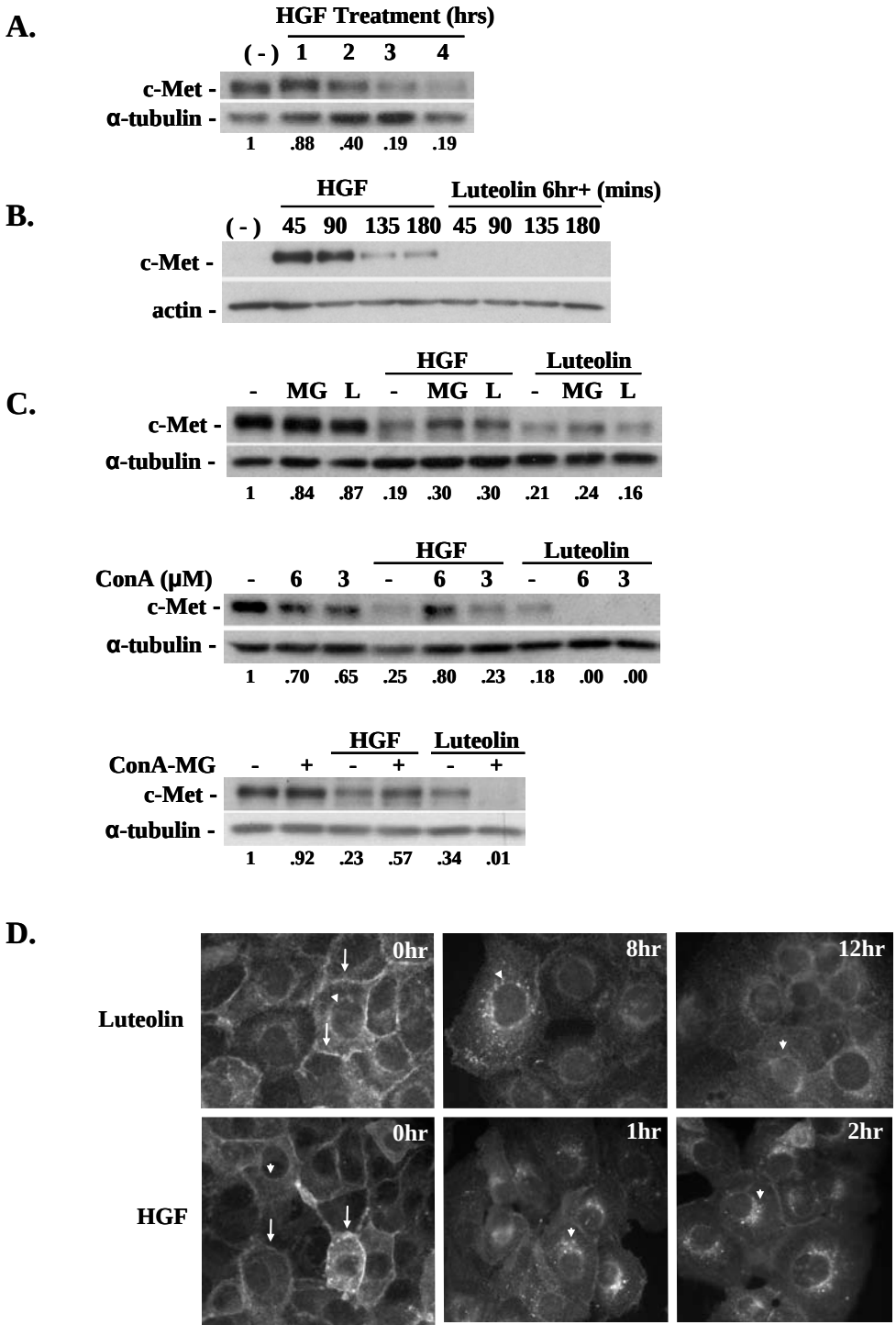
CHAPTER 1 FIGURE



CHAPTER 1 FIGURE LEGEND

Fig. 6. Luteolin downregulates c-Met levels independent of the lysosomal or proteosomal pathways. (**A)** DU145 cells were incubated alone (-) or treated with HGF (33ng/ml) for 1, 2, 3, or 4 hours. **(B)** DU145 cells were incubated alone (-), treated with HGF for 45, 90, 135, or 180 minutes, or treated with 25 μM luteolin for 6 hours plus 45, 90, 135, or 180 minutes. **(C top panel)** Cells were pretreated with DMSO or luteolin for 5 hours prior to culture alone (-) or with 300 nM MG132 (MG) or 20 μM lactacystin (L) for 4 hours with or without HGF stimulation. **(middle panel)** Cells were treated as described above, but using 6 μM or 3 μM concanamycin A (ConA), or **(bottom panel)** 300nM MG132 and 6μM concanamycin A in combination (ConA-MG). For each, whole cell lysates were analyzed by western blot analysis using the indicated antibodies. Actin or tubulin was used as a load control. Densitometry was performed on appropriate blots and shown as fold change of control. **(D)** DU145 cells were treated with 25uM luteolin for 8 and 12 hours or with HGF for 1 or 2 hours to stimulate loss of total c-Met. Cells were then fixed and stained with a c-Met specific primary antibody and fluorescently-labelled secondary antibody. Representative images are shown. Arrowheads point out perinuclear distribution and arrows show the cell membrane periphery.

CHAPTER 1 FIGURE

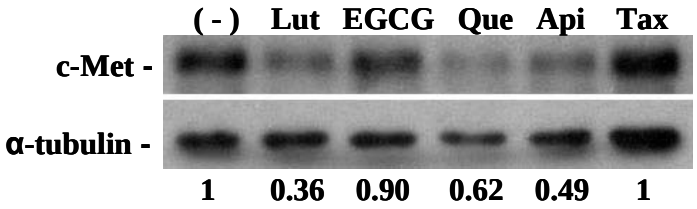


CHAPTER 1 SUPPLEMENTARY FIGURE LEGEND

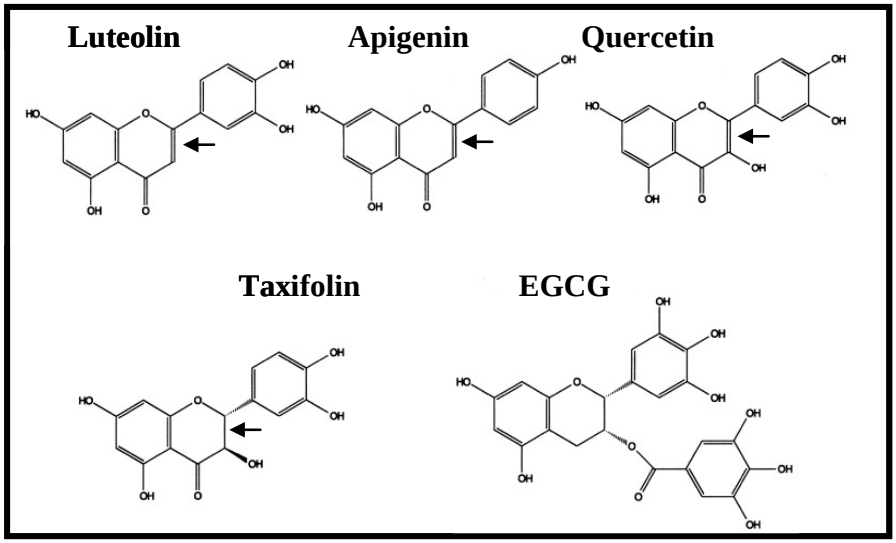
Supplemental Fig. 1. **c-Met Down regulation is a property common to several phytochemicals. (A)** DU145 cells were cultured alone (-) or with 25 μM each of luteolin (Lut), quercetin (Que), apigenin (Api), taxifolin (Tax), or 65 μM EGCG for 9 hours. Densitometric analysis was also performed to determine fold change. (**B)** Schematic of the chemical structures of these phytochemicals. Arrows indicate the double bond common to luteolin, quercetin, and apigenin; but not taxifolin.

CHAPTER 1 SUPPLEMENTARY FIGURE

A.



B.



REFERENCES

1. Gupta, G.P. and J. Massagué, Cancer Metastasis: Building a Framework. Cell, 2006. **127**(4): p. 679-695.
2. Thiery, J.P., Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, 2002. **2**(6): p. 442-454.
3. Birchmeier, C., et al., Met, metastasis, motility and more. Nat Rev Mol Cell Biol, 2003. **4**(12): p. 915-925.
4. Gentile, A., L. Trusolino, and P. Comoglio, The Met tyrosine kinase receptor in development and cancer. Cancer and Metastasis Reviews, 2008. **27**(1): p. 85-94.
5. Ernst Lengyel, et al., C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. International Journal of Cancer, 2005. **113**(4): p. 678-682.
6. Jeffers, M., et al., Activating mutations for the Met tyrosine kinase receptor in human cancer. Proceedings of the National Academy of Sciences, 1997. **94**(21): p. 11445-11450.
7. Knudsen, B.S., et al., High expression of the Met receptor in prostate cancer metastasis to bone. Urology, 2002. **60**(6): p. 1113-1117.
8. Humphrey, P.A., et al., Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. Am J Pathol, 1995. **147**(2): p. 386-96.

9. Engelman, J.A., et al., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science, 2007. **316**(5827): p. 1039-43.

10. Erdman, J.W., Jr., et al., Flavonoids and Heart Health: Proceedings of the ILSI North America Flavonoids Workshop,. J. Nutr., 2007. **137**(3): p. 718S-737.

11. Surh, Y.-J., Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer, 2003. **3**(10): p. 768-780.

12. Adachi, S., et al., The Inhibitory Effect of (-)-Epigallocatechin Gallate on Activation of the Epidermal Growth Factor Receptor Is Associated with Altered Lipid Order in HT29 Colon Cancer Cells. Cancer Res, 2007. **67**(13): p. 6493-6501.

13. Menendez, J.A. and R. Lupu, Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer, 2007. **7**(10): p. 763-777.

14. Wang, H.Q., et al., Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene, 2005. **24**(22): p. 3574-3582.

15. Swinnen, J.V., et al., Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochemical and Biophysical Research Communications, 2003. **302**(4): p. 898-903.

16. Brusselmans, K., et al., Induction of Cancer Cell Apoptosis by Flavonoids Is Associated with Their Ability to Inhibit Fatty Acid Synthase Activity. J. Biol. Chem., 2005. **280**(7): p. 5636-5645.

17. Rice-Evans, C.A., N.J. Miller, and G. Paganga, Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine, 1996. **20**(7): p. 933-956.

18. Bigelow, R.L.H. and J.A. Cardelli, The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF//Met signaling in immortalized and tumorigenic breast epithelial cells. Oncogene, 2006. **25**(13): p. 1922-1930.

19. Briaud, I., et al., Lipotoxicity of the Pancreatic {beta}-Cell Is Associated With Glucose-Dependent Esterification of Fatty Acids Into Neutral Lipids. Diabetes, 2001. **50**(2): p. 315-321.

20. Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer, Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol, 2005. **6**(1): p. 56-68.

21. Stambolic, V., et al., Negative Regulation of PKB/Akt-Dependent Cell Survival by the Tumor Suppressor PTEN. Cell, 1998. **95**(1): p. 29-39.

22. McMenamin, M.E., et al., Loss of PTEN Expression in Paraffin-embedded Primary Prostate Cancer Correlates with High Gleason Score and Advanced Stage. Cancer Res, 1999. **59**(17): p. 4291-4296.

23. Kuhajda, F.P., et al., Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc Natl Acad Sci, 2000. **97**(7): p. 3450-4.

24. Graff, J.R., et al., Targeting the Eukaryotic Translation Initiation Factor 4E for Cancer Therapy. Cancer Res, 2008. **68**(3): p. 631-634.

25. Abella, J.V., et al., Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol, 2005. **25**(21): p. 9632-45.

26. Hammond, D.E., et al., Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene, 2001. **20**(22): p. 2761-70.

27. Kermorgant, S., D. Zicha, and P.J. Parker, Protein Kinase C Controls Microtubule-based Traffic but Not Proteasomal Degradation of c-Met. J. Biol. Chem., 2003. **278**(31): p. 28921-28929.

28. Hoffmann, K.M., et al., Gastrointestinal Hormones Cause Rapid c-Met Receptor Down-regulation by a Novel Mechanism Involving Clathrin-mediated Endocytosis and a Lysosome-dependent Mechanism. J. Biol. Chem., 2006. **281**(49): p. 37705-37719.

29. Jeffers, M., et al., Degradation of the Met tyrosine kinase receptor by the ubiquitin- proteasome pathway. Mol. Cell. Biol., 1997. **17**(2): p. 799-808.

30. liShimizu, M., et al., (-)-Epigallocatechin Gallate and Polyphenon E Inhibit Growth and Activation of the Epidermal Growth Factor Receptor and Human Epidermal Growth Factor Receptor-2 Signaling Pathways in Human Colon Cancer Cells. Clin Cancer Res, 2005. **11**(7): p. 2735-2746.

31. Lee, W.-J., et al., Inhibitory effect of luteolin on hepatocyte growth factor/scatter factor-induced HepG2 cell invasion involving both MAPK/ERKs and PI3K-Akt pathways. Chemico-Biological Interactions, 2006. **160**(2): p. 123-133.

32. Hendriks, J.J.A., et al., Flavonoids Influence Monocytic GTPase Activity and Are Protective in Experimental Allergic Encephalitis. J. Exp. Med., 2004. **200**(12): p. 1667-1672.

33. Ridley, A.J., et al., Cell Migration: Integrating Signals from Front to Back. Science, 2003. **302**(5651): p. 1704-1709.

34. Agullo, G., et al., Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. Biochemical Pharmacology, 1997. **53**(11): p. 1649-1657.

35. Way, T.-D., M.-C. Kao, and J.-K. Lin, Degradation of HER2/neu by apigenin induces apoptosis through cytochrome c release and caspase-3 activation in HER2/neu-overexpressing breast cancer cells. FEBS Letters, 2005. **579**(1): p. 145-152.

36. Chiang, C.-T., T.-D. Way, and J.-K. Lin, Sensitizing HER2-overexpressing cancer cells to luteolin-induced apoptosis through suppressing p21WAF1/CIP1 expression with rapamycin. Mol Cancer Ther, 2007. **6**(7): p. 2127-2138.

37. Medes, G., A. Thomas, and S. Weinhouse, Metabolism of Neoplastic Tissue. IV. A Study of Lipid Synthesis in Neoplastic Tissue Slices in Vitro. Cancer Res, 1953. **13**(1): p. 27-29.

38. Huo, H., et al., Lipid Rafts/Caveolae Are Essential for Insulin-like Growth Factor-1 Receptor Signaling during 3T3-L1 Preadipocyte Differentiation Induction. J. Biol. Chem., 2003. **278**(13): p. 11561-11569.

39. Wang, L., et al., Effects of lipid rafts on signal transmembrane transduction mediated by c-Met. Zhonghua Gan Zang Bing Za Zhi, 2008. **16**(6): p. 449-52.

40. Pike, L.J., Growth factor receptors, lipid rafts and caveolae: An evolving story. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2005. **1746**(3): p. 260-273.

41. Nath, D., et al., Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. J Cell Sci, 2001. **114**(6): p. 1213-1220.

42. Way, T.-D., M.-C. Kao, and J.-K. Lin, Apigenin Induces Apoptosis through Proteasomal Degradation of HER2/neu in HER2/neu-overexpressing Breast Cancer Cells via the Phosphatidylinositol 3-Kinase/Akt-dependent Pathway. J. Biol. Chem., 2004. **279**(6): p. 4479-4489.

43. Vazquez-Martin, A., et al., Pharmacological blockade of fatty acid synthase (FASN) reverses acquired autoresistance to trastuzumab (Herceptin by transcriptionally inhibiting 'HER2 super-expression' occurring in high-dose trastuzumab-conditioned SKBR3/Tzb100 breast cancer cells. Int J Oncol, 2007. **31**(4): p. 769-76.

44. Menendez, J.A., et al., Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proceedings of the National Academy of Sciences, 2004. **101**(29): p. 10715-10720.

45. Bandyopadhyay, S., et al., FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. 2005. **24**(34): p. 5389-5395.

CHAPTER 2

Palmitoylation regulates the trafficking and stability of c-Met

ABSTRACT

c-Met is a receptor tyrosine kinase whose downstream signal transduction can promote both mitogenic and motogenic phenotypes involved in tissue development and cancer biology. Previous findings from our laboratory have led us to identify a novel mechanism by which FASN activity regulates c-Met expression. Our work has determined that inhibition or shRNA knockdown of FASN results in a post-translational downregulation of already synthesized c-Met protein.

Based on these findings, we have acquired convincing data that the c-Met receptor tyrosine kinase is palmitoylated and that this palmitoylation regulates its stability. Inhibition of palmitoylation reduced the expression of c-Met in multiple cancer cell lines. This protein loss occurred post-transcriptionally and was associated with accumulation of c-Met in Golgi compartments. Using inhibitors to a number of internalization pathways, as well as surface biotinylation studies, confocal microscopy, and metabolic-ortholog labeling we determined that inhibition of palmitoylation reduces the stability of newly synthesized c-Met, as opposed to inducing internalization and degradation. Moreover, both an acyl-biotin exchange technique and a click-chemistry based palmitate-labeling protocol suggested c-Met itself is palmitoylated. Observing palmitoylation kinetics provided evidence that c-Met is palmitoylated in the ER prior to cleavage of the 170 kd c-Met precursor into its mature 140 kd form. Taken together, these findings suggest inhibition of palmitoylation or FASN activity could be a novel target for preventing invasion and metastasis driven by c-Met overexpression.

INTRODUCTION

The c-Met receptor tyrosine kinase is involved in embryonic development, tissue remodeling, and when dysregulated, cancer progression [1]. Detailed by the Comoglio research group some 20 years ago, the c-Met protein is synthesized initially as a 170 kd single chain precursor that is co-translationally glycosylated and cleaved in the Golgi into a disulfide-linked α chain (50 kd) and βchain (140 kd) [2]. The receptor is trafficked through the Golgi to the plasma membrane where it is regulated through a steady-state of internalization and degradation, the kinetics of which are dramatically increased upon activation with ligand [3-6]. In addition to internalization, there is compelling evidence that the extracellular domain of c-Met can be cleaved by ADAM (a disintegrin and metalloproteinase) family proteases and shed, leaving the remaining transmembrane fragment to be cleaved by the presenilin-dependent γ-secretase complex and subsequently degraded through a proteasomal or lysosomal pathway [7-9]. Both internalization and shedding occur at a basal rate to maintain appropriate levels of c-Met or to desensitize the cells to the activated form. When bound by its cognate ligand, hepatocyte growth factor (HGF), the receptor homodimerizes at the plasma membrane promoting autophosphorylation and recruitment of downstream signaling components. The c-Met signaling cascade promotes growth, survival, and motility that is essential for development and tissue repair, but when unrestricted; due to gene amplification, activating mutations, or heightened ligand autocrine and paracrine secretion; the signaling pathway becomes oncogenic [10-14].

In the context of cancer, c-Met is frequently found to be overexpressed or mutated and this coincides with disease progression [14]. Overexpression of the receptor alone

can cause homodimerization and constitutive activation independent of ligand binding [15]. Aberrant, unregulated activation of the c-Met signaling pathways; including the PI3K, MAPK, and STAT pathways; can cause inappropriate/unrestricted proliferation and survival as well as heightened invasive potential [16-19]. Moreover, recent findings have shown a seemingly universal role for c-Met as a mediator of resistance to targeted therapies. A number of reports have identified c-Met's ability to circumvent targeted therapies, including the epidermal growth factor receptor (EGFR) inhibitor gefitinib in non-small cell lung cancer (NSCLC) and the BRAF inhibitor vemurafenib in melanoma, to reestablish sustained PI3K and MAPK signaling [20-24]. The relevance of c-Met in cancer is further highlighted by the recent success of c-Met inhibitors in clinical trials, particularly in c-Met diagnostic-positive patients [14, 25, 26]. Reducing the expression of or inhibiting the activity of c-Met is a clear target for preventing progression or therapeutic resistance of a number of cancers [27]. Understanding the regulation of c-Met will provide additional means of restricting its expression and activity therapeutically.

Our laboratory has previously reported, and others more recently, a connection between fatty acid synthase (FASN) activity and c-Met expression and activity. Inhibition of FASN activity lowered total c-Met protein levels, and this reduction was prevented by the addition of exogenous palmitate suggesting the end-product of FASN activity is required for stability of c-Met [28, 29]. FASN activity produces fatty acids, most abundantly the 16-carbon saturated fatty acid palmitate, from the simple precursors acetyl- and malonyl-CoA. FASN protein is expressed during embryonic development and in the adult liver and lactating breast, but at low to undetectable levels in most

tissues. In normal tissue, essential fatty acids are provided by a normal diet. However, in most cancers, FASN expression is elevated and levels often progressively correlate with disease stage. The explanations for this elevated expression are broad and include the requirement of lipids for membrane biogenesis, the modulation of cellular redox states and anaplerotic reactions, and the addiction to acylated signaling proteins that drive cancer proliferation and metastasis [30-34]. It has been demonstrated that FASN-derived *de novo* fatty acids exhibit a level of singularity in cellular usage over dietary fatty acids. For example, the Swinnen laboratory has shown that FASN-derived lipids are selectively partitioned into specialized plasma membrane domains of proliferating cancer cells [35]. In addition, the *de novo* synthesis of lipids is important for regulation of proteins that drive cancer progression [29, 36, 37].

Several groups have shown that inhibition of FASN activity can inhibit palmitoylation of proteins. Moreover, it seems that levels of FASN expression can modulate the palmitoylation of proteins and therefore their activity *in vivo* [37-39]. Palmitate is "activated" to palmitoyl-CoA by acyl-CoA synthetases before it can be bound to proteins by palmitoyl acyltransferases (PATs) via amide (N-terminal) or thioester (Cysteine-linked S-palmitoylation) bonds. Palmitoylation and other similar lipid-modifications, such as myristoylation (14-carbon amide-linked myristate) and prenylation (cysteine-linked cholesterol isoprene intermediates farnesyl or geranylgeranyl pyrophosphate), have been and continue to be candidates for drug targets [40, 41]. A number of cancer-related signaling proteins require palmitoylation for their spatial regulation including estrogen receptor-alpha, H- and N-Ras, caveolin-1 and most recently Rac1, just to name a few; but little has been shown for single-transmembrane spanning

receptor tyrosine kinases [41-45]. There is no strong consensus sequence for S-palmitoylation making predicting palmitoylated proteins challenging. In addition, the difficulties of traditional radiolabeling experiments including long exposure times and low detection limits has hampered the study of protein acylation [46, 47]. Given our observations, and previous reports that showed inhibition of FASN can inhibit palmitoylation, we hypothesized that either c-Met itself is palmitoylated or that other proteins that regulate its stability require palmitoylation. Herein, we sought to determine if there was a direct role for palmitoylation in c-Met protein expression and/or stability. Our findings suggest c-Met is, in fact, palmitoylated in the ER prior to its proteolytic processing, and that this lipid modification is required for stability and trafficking to the plasma membrane.

MATERIALS AND METHODS

Cell Culture and Reagents

All cell lines used were obtained from ATCC. DU145, H1993, AU565, and HCC1806, cells were maintained in RPMI-1640 media containing 10% FBS and MDA-MB-231 cells were maintained in DMEM (Cellgro, Herndon, VA, USA) containing 10% FBS (Gemini, CA, USA). Cells were kept at 37ºC with 5.0% CO_2. Monensin and brefeldin A were obtained from Biolegend (San Diego, CA, USA). C75, 2-hydroxymyristic acid, and 17-Octadecynoic Acid were obtained from Caymen Chemicals (Ann Arbor, MI, USA). Cycloheximide, recombinant EGF, geranylgeranyltransferase inhibitor, farnesyltransferase inhibitor and 2-Bromopalmitate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Click-It reaction buffers, azido-homoalanine, azide-palmitate, and methionine-free RPMI, were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant HGF was obtained from EMD Millipore (Darmstadt, Germany). Streptavidin Sepharose High Performance beads were obtained from GE Healthcare (Pittsburgh, PA).

Western blotting

Cells were seeded to 70% confluency in 24-well plates before treatments. Inhibitors were spiked into the media and incubated for the indicated times prior to cell lysis. Lysates were taken in boiling laemmli buffer with β-mercaptoethanol (BME) and boiled for 5 minutes. Samples were analyzed by SDS-PAGE and blotted with the indicated primary antibodies: c-Met CVD13 (Invitrogen), Integrin β4 H-101, Ron- β C-20, and EGFR 1005

(Santa Cruz Biotechnology, Santa Cruz, CA, USA), and alpha-tubulin (Neomarkers, Fremont, CA, USA). High sensitivity streptavidin-HRP was obtained from Pierce Thermo Scientific (Rockford, IL, USA). Blots were subsequently probed with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Pittsburgh, PA), and detection was acquired with ECL (Amersham Biosciences).

Confocal microscopy

Cells were grown to 50% confluency on glass coverslips in a 6-well plate. Following treatments cells were fixed in 4% PFA and washed twice with PBS and blocked for 30 minutes in 10% donkey serum PBS with .1% saponin (DSP). Primary antibodies: were added simultaneously in DSP for 2 hours at room temperature. After washing three times with PBS, secondary antibodies: were added in DSP for 2 hours at room temperature. Coverslips were washed again three times with PBS and then mounted on coverslips with DAPI slowfade (Invitrogen). Representative images were taken on a Leica TCS SP5 Confocal Microscope at 60x magnification with oil immersion. Representative images are shown as enhanced using ImageJ software.

Click Chemistry Palmitoylation Assays

Cell lines were grown to 80% confluency in 10cm dishes. All dishes were pretreated with growth media containing 10% fatty acid-free FBS (Gemini) with or without vehicle or treatment for indicated times. Media is replaced with fresh fatty acid-free growth media containing alkyne-labeled (Odya, Cayman) palmitic acid or azide-labeled (AZ-Palm, Invitrogen) that was sonicated for 5 minutes to improve solubility with or without

treatments. Cells were incubated for 4 hours to allow incorporation of palmitate orthologs. Cells lysates were taken in NP-40 lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) containing protease cocktail tablets (Roche) at 4 ^{o}C. Lysates are processed by 30 minutes of end-over-end rotation at 4 ^{o}C followed by removal of debris by centrifugation at 13,000xg for 5 minutes. Protein concentrations were determined and 200 µg of each sample were methanol-chloroform precipitated. Precipitated protein is resuspended in 50 µl of 1% SDS reaction buffer (50 mM Tris-HCL, 1% SDS, pH 8.0) and the click chemistry reaction was performed according to the manufacturer's protocol using the Click-iT reaction buffer kit and corresponding biotin-based detection reagent (Invitrogen). Following linkage of the palmitate ortholog to biotin, protein is again precipitated and resuspended in 100 µl of 1% SDS. Once protein is solubilized, SDS is diluted out with 1 mL of NP-40 lysis buffer. Samples are precleared for 1 hour at 4 ^{o}C with A/G agarose beads and centrifuged at 13,000xg for 5 minutes (Santa Cruz). After preclear, 100 µl of prewashed streptavidin-conjugated sepharose beads (GE Healthcare) are added to each supernatant. Samples are rotated end-over-end overnight at 4 ^{o}C. IP samples are washed 5 times (50 volumes) in NP-40 lysis buffer, before protein is eluted with 30 µl of laemmli buffer with BME. Samples were analyzed by Western blot using antibody to c-Met (Invitrogen,).

Click Chemistry Nascent Protein Detection

Cell lines were grown to 70% confluency in 10 cm dishes. All dishes were incubated in Methionine-free growth media (Invitrogen) containing 10% FBS for 1 hour prior to treatments. Any pretreatment inhibitor was spiked into the well for the last 30 minutes of

the Methionine-starve period. Media was then replaced with fresh Methionine-free growth media containing 10% FBS containing with or without 30 µM azido-homoalanine (AHA) (Invitrogen) to label nascent proteins with or without indicated treatments. Processing of lysates, Click-Chemistry reaction, and immunoprecipitation of biotinylated protein was performed as detailed above.

Surface Kinetics

Cells were grown to 70% confluency in 10 cm dishes. Cells were washed once with 4 ^{o}C PBS to prevent membrane internalization. Surface-exposed proteins were biotinylated with .1mg/ml EZ-Link biotin (Invitrogen) in PBS at 4 ^{o}C for 15 minutes. Excess biotin was quenched and washed off with 3 5 minute washes of 100 mM glycine in PBS at 4 ^{o}C. All plates, except one complete strip control, were then incubated with 37 ^{o}C prewarmed serum-free media containing the indicated treatments for chase periods to allow internalization. After indicated time chase time periods, cell were washed 2 times with 4 ^{o}C PBS to halt the experiment. Remaining extracellular biotin was removed, from all but a no strip control, with MESNA buffer in PBS (100 mM MESNA, 100 mM NaCl, 1 mM EDTA, 50 mM TrisBase, .2% BSA, pH 8.6) for 3 5 minute washes at 4 ^{o}C. Residual MESNA was removed with 6 brief washes with 4 ^{o}C PBS. Cells lysates are taken in NP-40 lysis buffer containing protease cocktail tablets (Roche) at 4 ^{o}C. Processing of lysates and precipitation of biotinylated protein from equal protein samples was performed as detailed above. Equal protein was analyzed for c-Met by Western blotting to indicate amount of c-Met internalized over time.

Shedding

Cells were grown to 60% confluency in 10 cm dishes. Dishes were washed 2 times with room temperature PBS and then treated with 3.5 mls serum free media containing the indicated treatments: EGF 50, 100 ng/ml, HGF 33 ng/ml, C75 10/20 µM and incubated for the indicated time. Conditioned media was collected and washed cells were lysed in NP-40 lysis buffer. The Conditioned media was centrifuged at 2100 rpm for 5 minutes at 4^{o}C to pellet cell debris. Supernatant was transferred to a clean tube for TCA precipitation. Fresh 2% NaDeoxycholate was added 1:100, gently mixed and incubated at room temperature for 15 minutes. Fresh TCA was then added at 1:10, vortexed, and incubated at room temperature for 1 hour. The protein was centrifuged out at 5500 rpm for 10mins at 4 ^{o}C. The supernatant was aspirated and the pellet was allowed to dry. The pellet was then washed in 200 µl ice cold acetone, vortexed, and left on ice for an additional 15 minutes, followed by a repeat centrifugation at 5500 rpm for 10 minutes at 4 ^{o}C. Supernatants were then aspirated and the pellet allowed to dry. 100 µl of laemmli buffer with BME was added to each protein pellet. Equal volumes were loaded and analyzed by SDS-PAGE blotting with the DL-21 antibody (Upstate) to an extracellular c-Met epitope. A representative blot from three independent experiments is shown.

RESULTS

Inhibition of palmitoylation lowers total c-Met protein levels

To determine if c-Met protein expression is affected by palmitoylation, we used a common palmitoylation inhibitor 2-bromopalmitate (2BP). Although the exact mechanism of action for 2BP has not been definitively shown, most likely through competitive inhibition or the formation of a nonmetabolizable intermediate to interfere with PAT or ACSVL activity, it clearly prevented the palmitoylation of numerous example proteins [48-50]. DU145 prostate cancer cells were treated with 100 μM 2BP for time points through 6 hours and Western blot analysis was performed to determine if c-Met protein levels were affected. c-Met levels were reduced over the 6 hour time course with a significant reduction beginning at 2 hours (Fig. 1A). 2BP did not affect FASN activity as determined by analysis of ^{14}C-acetate incorporation into lipids (Supp. Fig 1A).

To determine if the effect of 2BP was specific to inhibition of a lipid modification involving the 16-carbon saturated fatty acid palmitate rather than a general inhibitory effect of lipid analogs or inhibitors of other lipid modifications, we treated DU145 cells with an inhibitor of myristoylation (OH-Myr), farnesylation (FTI), or geranylgeranylation (GGTI). As indicated by Western blotting, treatment with these inhibitors at maximum nontoxic concentrations did not reduce the expression of c-Met (Fig. 1A). In addition, we tested whether the effect of 2BP was unique to c-Met or if a similar effect could be observed for other membrane spanning proteins. Although there was a 50% reduction in the EGFR receptor at the latest time point (Fig 1B), c-Met was downregulated more rapidly and more completely. Interestingly, neither integrin β4 nor RON, a receptor

tyrosine kinase of the Met family, was substantially downregulated in response to 2BP treatment through 6 hours. Integrin β4 is known to be palmitoylated and there are several reports delineating a mechanism for integrin β4 regulation through dynamic palmitoylation; however, as observed here, there is no previous report that inhibition of palmitoylation causes a loss of the protein, but merely a change in intracellular trafficking [51, 52]. There is no previously reported evidence for direct palmitoylation of EGFR or RON nor is there evidence for these proteins in their native form being affected by inhibition of palmitoylation.

To visualize the effect of 2BP on c-Met localization, DU145 cells were treated with 2BP for 3 or 6 hours, fixed and fluorescently labeled with antibody to c-Met. With increasing time of 2BP treatment, c-Met accumulated perinuclear and slowly diminished from the plasma membrane, most strikingly at 6 hours (Fig. 1C).

Downregulation of c-Met in response to inhibition of palmitoylation occurs post-translationally

Given the kinetics of c-Met loss in response to inhibition of palmitoylation we sought to determine at what level c-Met biosynthesis and/or maintenance was being affected. Quantitative real-time (not shown) and rtPCR were performed on DU145 cells treated with or without 2BP for 6 hours along with a parallel Western blot protein analysis (Fig. 2A). There was less than 10% change in the levels of c-Met mRNA concurrent with a greater than 50% reduction in c-Met protein indicating the downregulation of c-Met occurs post-transcriptionally. Rates of general protein synthesis and c-Met protein synthesis specifically were then analyzed to determine effects of 2BP

on translation. Using a click-chemistry based approach to detect the incorporation of the methionine ortholog, azido-homoalanine (AHA), into newly synthesized protein, it was determined that 2BP treatment did not have significant effects on general protein synthesis, nor did 2BP specifically reduce the rate of c-Met synthesis in particular (Fig. 2B and Supp. 2A, B). Taken together, these data indicate that it is more likely that 2BP was reducing the stability and therefore half-life of already synthesized c-Met protein.

Inhibition of palmitoylation disrupts the trafficking of c-Met and ultimately leads to loss of c-Met protein

The apparent reduced half-life of c-Met could be accounted for by an increase in the internalization and degradation of the plasma membrane c-Met pool and/or by a block in the trafficking of c-Met to the plasma membrane along the biosynthetic and secretory pathway. To distinguish these possibilities, we performed confocal microscopy on 2BP treated DU145 cells and looked for colocalization of c-Met with protein markers of different biosynthesis and trafficking sites including the endoplasmic reticulum (calnexin), the cis-Golgi (GM130), and early endosomes (EEA1). With increased time of 2BP treatment, we observed reduced c-Met staining at the plasma membrane and an accumulation near the nucleus. There was no colocalization with early endosomes, suggesting 2BP treatment did not induce rapid internalization; however there was clearly loss from the plasma membrane (Fig. 2C and Supp. 3A). In addition, there was no colocalization of c-Met at the ER, but there was distinct colocalization in the Golgi, suggesting that there was a block in the biosynthetic processing of c-Met in the Golgi which most likely leads to its degradation (Fig. 2C and Supp. 3B). Given the clear loss of

c-Met protein, we sought to determine the degradation mechanism responsible for the post-translational loss. DU145 cells were treated with 2BP for 2, 4, or 6 hours in the presence or absence of the lysosome acidification inhibitor concanamycin A (ConA) or the proteasome inhibitor MG132. Western blot analysis revealed that ConA actually had an additive effect with 2BP treatment, and MG132 only partially prevented 2BP-induced c-Met loss (Fig. 3A, B). To further address the loss of c-Met from the plasma membrane, we performed a surface biotinylation assay to monitor the kinetics of c-Met internalization. Due to constraints of 2BP toxicity in serum-free media, it was necessary to use the FASN inhibitor C75 to block palmitoylation. We have also shown C75 lower c-Met levels, likely through inhibition of palmitoylation [28]. Monitoring the internalization of c-Met revealed that inhibition of palmitoylation, through FASN inhibition, strikingly reduced the rate of c-Met internalization whereas ligand stimulation as a positive control greatly increased the rate of internalization over basal levels (Fig. 3C). In addition, loss of c-Met in response to C75 or 2BP treatment was not prevented by any means from an extensive list of strategies to block common pathways of internalization and degradation (Supp. Table 1).

These experiments along with observations from microscopy and surface biotinylation would suggest that inhibition of palmitoylation was possibly causing shedding of plasma membrane associated c-Met. To test this possibility, we collected conditioned media from DU145 cells treated with either HGF or EGF as positive controls or C75 to inhibit palmitoylation to detect possible increases in the previously published 90 kd shed c-Met ectodomain fragment. Using an antibody to an extracellular epitope of

c-Met it was determined that there was a substantial increase in the shed 90 kd c-Met fragment detected upon inhibition of FASN or palmitoylation with C75 (Fig. 3D).

c-Met is S-palmitoylated

Given the potent effects of palmitoylation inhibition on c-Met levels, we tested if c-Met protein itself was palmitoylated or alternatively, if other proteins involved in maintaining the proper trafficking and stability of c-Met required palmitoylation for function. We initially utilized an acyl-biotin exchange technique to determine if any cysteine residues were modified via a thioester bond which would suggest S-palmitoylation. In brief, c-Met protein was immunoprecipitated from DU145 cells, all unmodified free cysteine residues were covalently blocked by N-ethylmaliamide, thioester-linked cysteine modifications were removed with hydroxylamine treatment, and biotin was then linked to any now-free cysteine residues [46]. These samples were separated by SDS-PAGE and blotted with streptavidin-HRP to detect the presence of biotinylated c-Met. Western blot analysis indicated that the initial block of unbound cysteine residues was complete ($-NH_2OH$) and that c-Met was biotinylated only when bound cysteines were reduced and exposed to biotin binding ($+NH_2OH$) (Fig. 4A).

To further support the idea that c-Met as a palmitoylated protein, we used a more flexible click chemistry-based approach with palmitate orthologs azide-palmitate (AZ-Palm) or alkyne-linked 17-Octadecynoic acid (Odya) to detect palmitoylation [53]. Palmitoylation of c-Met was observed in every cell line assessed including the H1993 lung cancer cell line as well as AU565, HCC1806, and MDA-MB-231 breast cancer cell line (Fig. 4B, D). c-Met labeling was equally detectable using two distinct click-based

palmitate orthologs (Az-palm and Odya, Fig. 4B, C), and exhibited time dependence (5 min vs 5 hrs, Fig. 4B) providing additional support for specificity of this technique. In panel 4C, DU145 cells were treated with 200 μM Az-Palm or unlabeled palmitate (mock) for 4 hours to allow palmitate to be incorporated into palmitoylated proteins. Equal protein from each sample was processed through the click reaction to covalently link biotin to the palmitate ortholog, biotinylated protein was precipitated, and samples could then be reduced and immunoblotted for c-Met. Included in this, to emphasize specificity, were samples lysates divided into those processed through the click reaction (RXN) versus those that were not (No RXN) and samples that were processed only after thioester bonds were broken by hydroxylamine treatment (NH_2OH) removing any cysteine-linked palmitate. Western blot analysis showed that labeled palmitate was incorporated into c-Met in a manner that was sensitive to a reducing agent, again suggesting S-palmitoylation (Fig. 4C). The H1993 lung cancer cell line has amplified levels of c-Met compared to the relatively low levels of c-Met expression in DU145 cells, and were used for several experiments described below that were hampered by the limited expression of c-Met overtime in DU145 cells. Using protein lysates from H1993 lung cancer cells incubated with Odya, c-Met was immunoprecipitated, reduced with TCEP to break disulfide bonds while maintaining the thioester linked palmitate, and then blotted with streptavidin-HRP after SDS-PAGE to show the reverse approach of detection holds true (Fig. 4E).

To show that 2BP treatment was inhibiting the palmitoylation of c-Met we incubated H1993 lung cancer cells with Odya over time in the presence or absence of 100 μM 2BP. Lysates were processed through the click reaction, biotinylated proteins were precipitated, and samples were immunoblotted to observe palmitoylation of c-Met.

Western blot analysis revealed that c-Met incorporated labeled palmitate over time and that 2BP treatment prevented this palmitoylation (Fig. 4F).

c-Met palmitoylation occurs in the ER

Based on the compelling data that c-Met is palmitoylated, we investigated the dynamics of palmitoylation during the lifespan of c-Met. H1993 cells were first incubated with AHA, to label nascent protein, in the presence or absence of cycloheximide (CX, 10 μg/ml) to inhibit protein synthesis, brefeldin A (BF, 2 μM) to inhibit protein transport from the ER to the cis-Golgi, or monensin (MN, 2 μM) to prevent transport out of the Golgi network to the plasma membrane, in order to demonstrate their effect on c-Met protein. Early studies on the biosynthesis of c-Met determined that the protein was synthesized in a 170 kd precursor that is subsequently cleaved in the Golgi to the α- and β-chains that are linked via disulfide bonds. Under reducing conditions, the mature β-chain migrates at 140 kd by SDS-PAGE. Consistent with this, when H1993 cells were labeled with AHA at time points within 2 hours in combination with brefeldin A, it was shown, by Western blot, that the 170 kd c-Met precursor accumulated having not entered the Golgi for precursor cleavage. Furthermore, cycloheximide treatment effectively prevented new synthesis of c-Met, but monensin did not disrupt synthesis or processing of c-Met inside the Golgi (Fig. 5A).

The antibody to c-Met used for immunofluorescence analysis was unable to detect c-Met in its uncleaved precursor form. Exploiting this knowledge, DU145 cells were treated with reversible brefeldin A or monensin and stained to observe c-Met distribution. Perinuclear c-Met was not detected under brefeldin A treatment until the compound was

washed away allowing a bolus of c-Met to emerge processed in the Golgi (Supp. 4A). Monensin treatment, conversely, is shown to caused accumulation of c-Met within the Golgi (Supp. 4B). Prolonged treatment with either inhibitor leads to loss of c-Met staining from the plasma membrane, similar to what was observed with 2BP treatment (Supp. 4A, B).

With this information, we sought to determine the location and kinetics of c-Met palmitoylation. H1993 cells were labeled with Odya for time points within 2 hours in the presence or absence of CX, BF, or MN and Western blot analysis was performed to observe palmitoylated c-Met. In the presence of CX treatment there is no detected level of c-Met palmitoylation above the IP background (No Odya). Treatment with BF showed the 170 kd precursor form of c-Met was palmitoylated and that c-Met palmitoylation must therefore occur in the ER. MN treatment did not appear to affect the kinetics of c-Met palmitoylation under basal growth conditions (Fig. 5B). As detailed with other proteins, it is possible that the dynamics of c-Met palmitoylation are altered in response to its ligand, HGF. To test this, we incubated DU145 cells for 2 hours (T0) with Odya to have a bolus of biotinylated c-Met at the plasma membrane. Without changing the media, cells were chased for additional 20 or 40 minutes in the presence or absence of HGF (33 ng/ml). As indicated by Western blot analysis, HGF did not cause a noticeable increase or decrease in the amount of palmitoylated c-Met (Fig. 5C).

DISCUSSION

Our laboratory had previously determined that inhibition of or shRNA knockdown of FASN can cause the post-translational downregulation of c-Met. Knowing that FASN activity can regulate palmitoylation of proteins, we sought to determine if c-Met expression was regulated either directly or indirectly by protein palmitoylation. Using the palmitoylation inhibitor 2BP, we found that c-Met was downregulated over a time course of treatment, and other similar inhibitors of lipid modifications did not have this effect. This experiment highlights the sensitivity of c-Met to inhibition of palmitoylation as compared to acylation by the structurally similar 14-carbon myristate or prenylation by farnesyl or geranylgeranyl groups. In addition, the downregulation of c-Met by 2BP treatment was unique compared to several other transmembrane proteins analyzed including EGFR, integrin β4, and RON which were not substantially downregulated within early time points. Integrin β4 is known to be palmitoylated and there are several reports delineating a mechanism for integrin β4 regulation through dynamic palmitoylation; however, as observed here, there is no evidence that inhibition of palmitoylation causes a loss of the protein, but merely a change in intracellular trafficking [51, 52]. There is no previously reported evidence for direct palmitoylation of EGFR or RON nor is there evidence for these proteins in their native form being affected by inhibition of palmitoylation. However, protein palmitoylation has been shown to regulate the activity of a long list of proteins often through affecting trafficking and stability.

Our data suggest c-Met expression is sensitive to the effects of blocked palmitoylation, and in order to understand this effect we examined its transcription,

synthesis, and intracellular localization. Using immunofluorescence confocal microscopy we observed that c-Met expression was reduced from the cell surface and led to the accumulation of c-Met in a perinuclear compartment following inhibition of palmitoylation. These results could indicate that 2BP caused the internalization and degradation of c-Met from the cell surface, or that 2BP allowed a basal level of surface c-Met loss but reduces the rate of nascent c-Met from reaching the plasma membrane. PCR analysis indicated that there was very little change in the mRNA levels of c-Met at times of 2BP treatment in which parallel Western blotting showed greater than 50% c-Met protein loss. These data indicate the reduction of c-Met occurs post-transcriptionally either from a reduced rate of c-Met protein translation or an increased rate of degradation. Using a click chemistry-based approach for monitoring nascent protein synthesis, we found that neither general protein synthesis, nor c-Met synthesis was slowed by 2BP treatment over a 6 hour time course. These data were compelling evidence that the effect blocking palmitoylation had on c-Met expression was at a post-translational level, similar to what we have observed previously with FASN inhibition, and that 2BP treatment was not having a global effect on protein synthesis.

c-Met is known to be processed into its mature form in the ER and trafficked through the Golgi to the plasma membrane where it is subject to basal rates of downregulation [2]. We performed immunofluorescence confocal microscopy studies to identify the changes in c-Met intracellular localization upon 2BP treatment. We used colocalization markers to early endosomes, the ER, and the cis-Golgi to determine the compartment in which c-Met was accumulating during 2BP treatment. We found that the bulk of the perinuclear c-Met colocalized with the cis-Golgi marker. This suggests that

when palmitoylation is inhibited, c-Met is blocked from continuing through its normal biosynthetic route and that possibly this block eventually causes it to be shunted off for degradation.

It was also apparent that there was marked reduction of c-Met staining at the cell surface. This plasma membrane-associated population of c-Met could be internalized and degraded at an increased rate or simply the normal basal rate; or alternatively, could be lost through ectodomain shedding. Our results from surface biotinylation assays indicated that the kinetics of c-Met internalization were greatly reduced in response to FASN inhibition compared to basal or HGF-stimulated rates of internalization. Given the aforementioned findings that clearly show loss of c-Met from the cell surface, these unexpected results indicate the receptor may be lost from the surface by ectodomain shedding. Probing for the 90 kd c-Met ectodomain following treatment with FASN inhibitor C75, we were able to detect ectodomain fragments at elevated levels, but not as abundant as following EGF treatment, which is known to potently stimulate c-Met shedding. 2BP is highly toxic to DU145 cells under serum free conditions and therefore the FASN inhibitor C75 was required under the conditions needed for this assay. However, there is evidence that FASN inhibition is most likely affecting c-Met through blocking palmitoylation and we believe them to be interchangeable in these studies. Taken together, the results suggest that inhibition of palmitoylation, either directly or through inhibition of FASN activity, causes a loss of c-Met surface expression, at least in part, through ectodomain shedding.

We also performed studies blocking degradation through the proteasomal and lysosomal pathways and were unable to significantly prevent 2BP-induced c-Met protein

loss. These being the major mediators of protein degradation in the cell it is difficult to accept that they do not play a role; although, multiple reports have detailed the complex paradoxical roles these two pathways have on the regulation of c-Met trafficking and stability. From this, it is reasonable that under conditions where FASN or palmitoylation is inhibited, c-Met pools in different intracellular locations are degraded through different, possibly compensatory, pathways. Consistently, and most importantly, inhibition of palmitoylation prevents nascent c-Met protein from refreshing the plasma membrane-expressed pool.

Given that palmitoylation regulated c-Met trafficking and stability so profoundly, we sought to determine if the c-Met protein was modified by the attachment of palmitate itself. A technique known as acyl-biotin exchange (ABE) has been used extensively to identify proteins with thioester-linked modifications. This method is somewhat specific to palmitoylation; however, it could include modifications such as myristoylation which can be thioester-linked at a cysteine residue, although it is more commonly through a co-translational amide linkage, or prenylation which commonly occurs via thioester linkage but it is typically directed by a c-terminal CAAX consensus sequence. Using ABE we were able to determine that c-Met is modified through a thioester-linked attachment. To support these data we performed experiments exploiting click chemistry to label palmitoylated proteins with a non-radioactive bio-orthogonal-labeled palmitate. This relatively new, yet thoroughly utilized technique allows for a much more rapid and tractable study of palmitoylated proteins while maintaining confirmed specificity. From these experiments, c-Met was shown to be palmitoylated in every cell line tested, with two different bioorthogonal reporters (azide and alkyne), and in a manner sensitive to

reducing agent treatment which signifies cysteine-linked S-palmitoylation. In addition, palmitoylation of c-Met was a time-dependent reaction defending the fact that it is not due to nonspecific post-lysis enzymatic activity. This is the first evidence of c-Met being palmitoylated in any cell type, as well as the only evidence for any oncogenic receptor tyrosine kinase being palmitoylated. We believe there is plentiful reproducible data supporting the accuracy of these results. To understand the kinetics and location of c-Met palmitoylation we used inhibitors to block steps along the biosynthetic path of c-Met. We found that cycloheximide treatment completely blocked palmitoylation of c-Met and brefeldin A treatment greatly reduced palmitoylation of the mature form of c-Met. Thus, it is likely that c-Met is palmitoylated prior to leaving the ER in its precleaved 170 kd precursor structure. In this model, palmitate would be attached to precursor protein in the ER, most likely catalyzed by a resident PAT, although the possibility of autoacylation cannot be ruled out. Palmitate would remain attached to one or more cysteine residue/s within the β-chain through cleavage in the Golgi and as it is trafficked to the plasma membrane. Given that monensin treatment had little effect on the amount of c-Met palmitoylated overtime, we would predict that neither association with the plasma membrane nor internalization and recycling kinetics control or are controlled by dynamic palmitoylation, but rather this is a stable attachment that remains throughout the duration of the molecule's life. In support of this, ligand stimulation, which is known to cause internalization of the receptor, did not modify the palmitoylation status of c-Met. Blocking palmitoylation did not seem to affect exit from the ER or cleavage in the Golgi, but it did impede exit of c-Met out of the Golgi through the secretory pathway. It is possible that the requirement of palmitoylation for c-Met to exit

the Golgi is related to trans-Golgi lipid raft structures [54, 55]. In the absence of palmitoylation, c-Met may not integrate into these documented lipid raft domains potentially required for its trafficking and would; therefore, accumulate until degraded, potentially through an ER-stress type mechanism. Extensive work will be required to test this hypothesis.

Our dependence on a general palmitoylation inhibitor somewhat limits the strength of our model; however, the highly specific data for the kinetics of palmitoylation using bio-orthologs reinforces its accuracy. It will be difficult to more closely define the specific influence of palmitoylation on c-Met regulation until the palmitoylation sites are identified and mutant forms developed.

The findings detailed in this paper reveal a novel post-translation modification that is required for the trafficking and stability of a clinically important receptor tyrosine kinase. There is an abundance of evidence implicating the c-Met receptor tyrosine kinase as a strong molecular target for cancer therapy, but it is also relevant in embryonic development, liver regeneration, and fibrosis. As of the writing of this paper, there are nearly two dozen specific and non-specific c-Met targeting therapies being evaluating in clinical trials for a variety of human tumors [1, 19]. Similarly, several normal and disease-state physiological processes including embryonic development, obesity, diabetes, and cancer are frequently associated with changes in the expression and activity of FASN [33]. Several reports have linked this association with changes in the acylation of proteins relevant to these processes. It is intriguing to conjecture that the changes in FASN expression can, in and of itself, regulate the acylation of proteins and; therefore, aberrant expression of FASN can have a causal role in certain pathology by dysregulating

protein trafficking and stability. With regard to c-Met, its connection with FASN may be due to a need for *de novo* palmitate to maintain its stability, particularly in the context of cancer-associated overexpression. Future work should address the possibility of therapeutically targeting FASN and acylation as a means of restricting disease-associated c-Met expression.

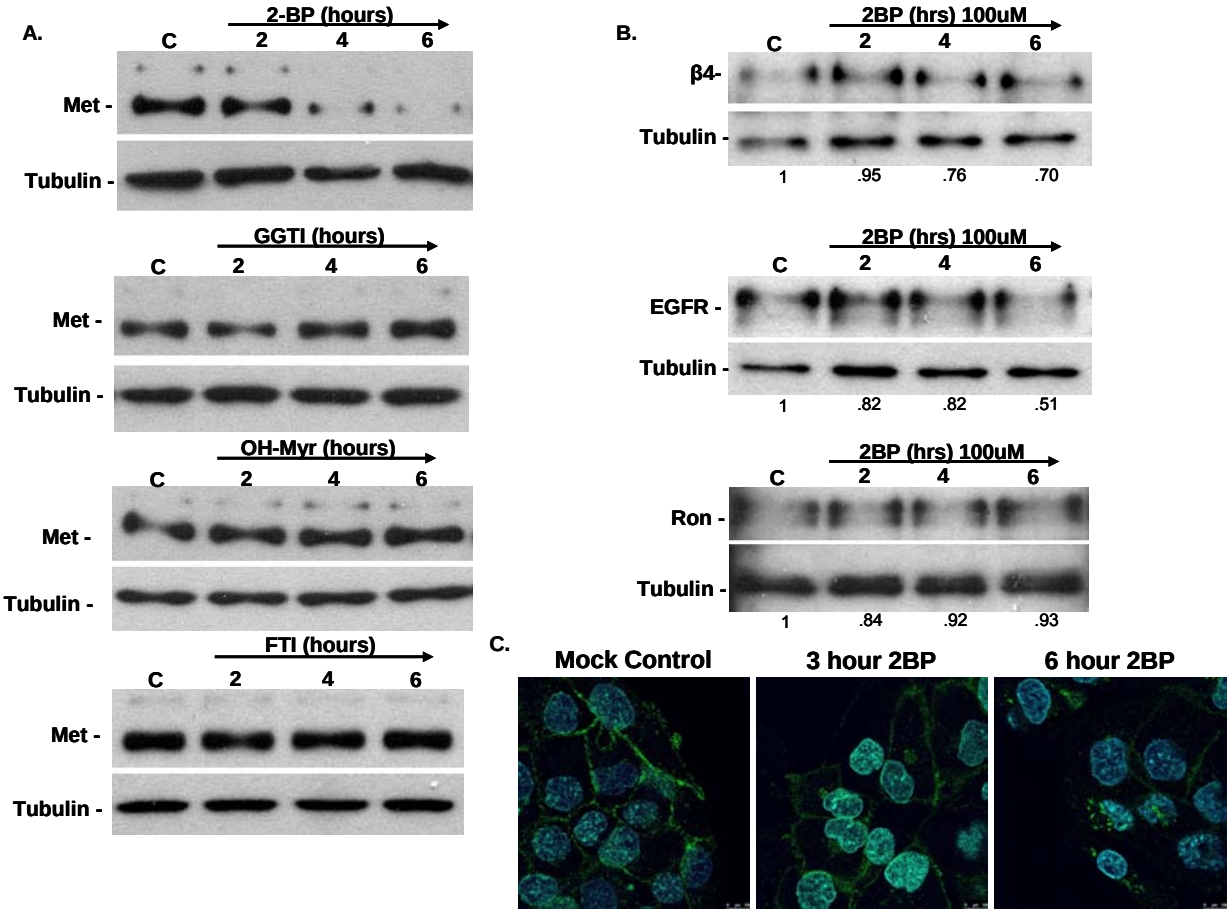
ACKNOWLEDGEMENTS

The authors would like to thank Dr. Marilyn Resh, Dr. Ruth Lupu, and Dr. Stephen Kridal for thoughtful insight and technical assistance with palmitoylation and fatty acid synthase activity assays. This work was supported through a Department of Defense predoctoral fellowship PC102179.

CHAPTER 2 FIGURE LEGEND

Figure 1. Inhibitors of palmitoylation, but not other lipid modifications, lower total c-Met protein levels. (A) DU145 prostate cancer cells were treated with DMSO or 100 μM 2BP, 100 μM OH-Myr, 100 μM GGTI, or 100 μM FTI for 2, 4, or 6 hours. Western blot analysis was performed to indicate levels of (A) c-Met or (B) EGFR, RON, or Integrin β4. Representative blots from three independent experiments are shown. (C) DU145 cells were treated with 2BP for 3 or 6 hours then fixed and stained for c-Met. Representative 60x confocal images from three independent experiments are shown.

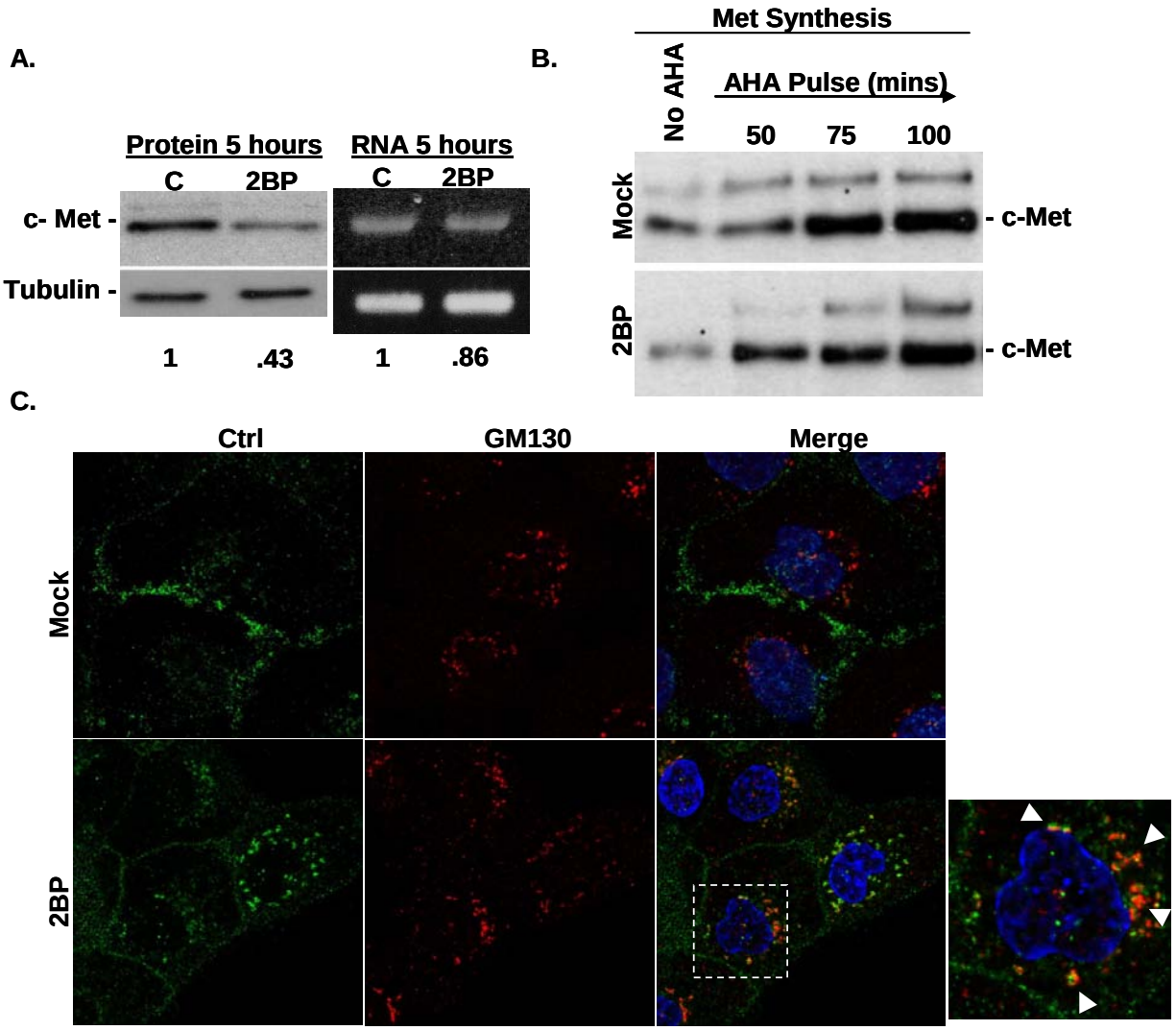
CHAPTER 2 FIGURE



CHAPTER 2 FIGURE LEGEND

Figure 2. 2BP treatment affects c-Met stability post-translationally and causes a trafficking block in the Golgi. (A) DU145 cells were treated with 100 μM 2BP for 5 hours. Western blot analysis was performed for each to determine levels of total c-Met protein following treatment. In addition, (A) RT-PCR was performed to indicate c-Met RNA levels following 2-BP treatment. (B) DU145 cells were incubated for 50, 75, or 100 minutes with azido-homoalanine in the presence or absence of 100 μM 2BP in order to label newly synthesized protein with biotin through a subsequent click chemistry-based reaction. Biotinylated protein was immunoprecipitated and levels of newly synthesized c-Met were identified by western blot analysis. The representative blots were run on the same gel, but are overlaid here for visual convenience. (C) DU145 cells were treated with 100 μM 2BP for 2.5 hours prior to fixing. Antibodies to c-Met (green) or the cis-Golgi marker GM130 (red) were used where indicated for immunofluorescence. Representative 60x confocal images from three independent experiments are shown.

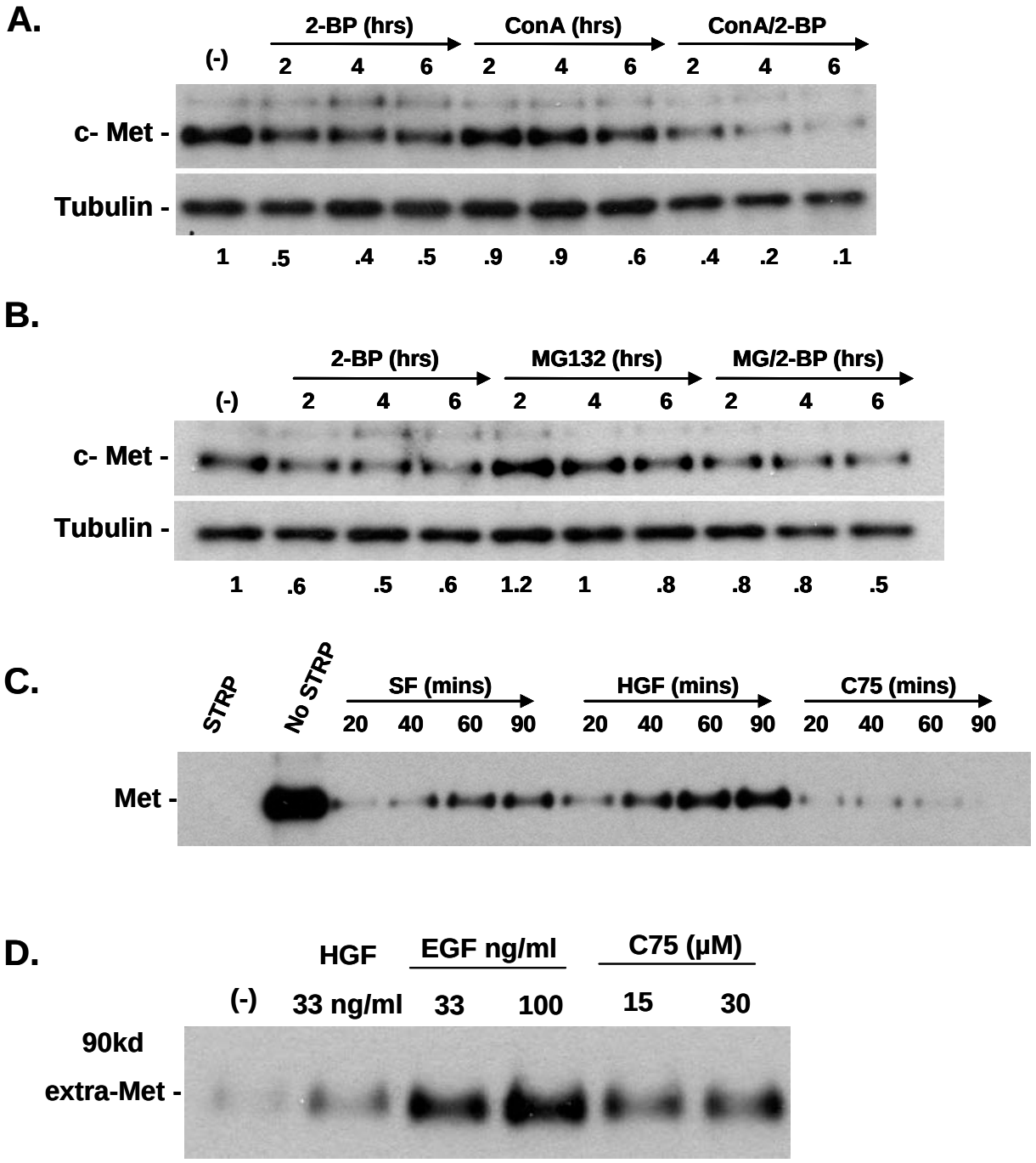
CHAPTER 2 FIGURE



CHAPTER 2 FIGURE LEGEND

Fig. 3. Inhibition of FASN or palmitoylation reduce c-Met levels independent of proteasomal and lysosomal degradation and may be, at least in part, lost through ectodomain shedding. DU145 cells were treated with 2-BP and an inhibitor of lysosomal acidification (A) ConA or a proteosome inhibitor (B) MG132 alone or in combination for 2, 4, or 6 hours. (C) A Surface biotinylation assay was performed to observe the kinetics of c-Met internalization in response to FASN inhibition. DU145 cells having been surface biotinylated were incubated at 37°C for the indicated time under basal serum free (SF) conditions, stimulated with HGF to show an increased internalization rate as a positive control, or with the FASN inhibitor C75. All proteins remaining on the cell surface after the indicated time were stripped off (STRP). Biotinylated protein was immunoprecipitated and internalized c-Met was identified by western blot analysis. (D) DU145 cells were treated with 33 ng/ml HGF, 33 or 100 ng/ml EGF, or 15 or 30 μM C75 for 8 hours. Conditioned media was collected and concentrated and Western blot analysis was performed to detect the 90kd ectodomain of c-Met. For each experiment, representative Western blot images from three independent experiments are shown.

CHAPTER 2 FIGURE

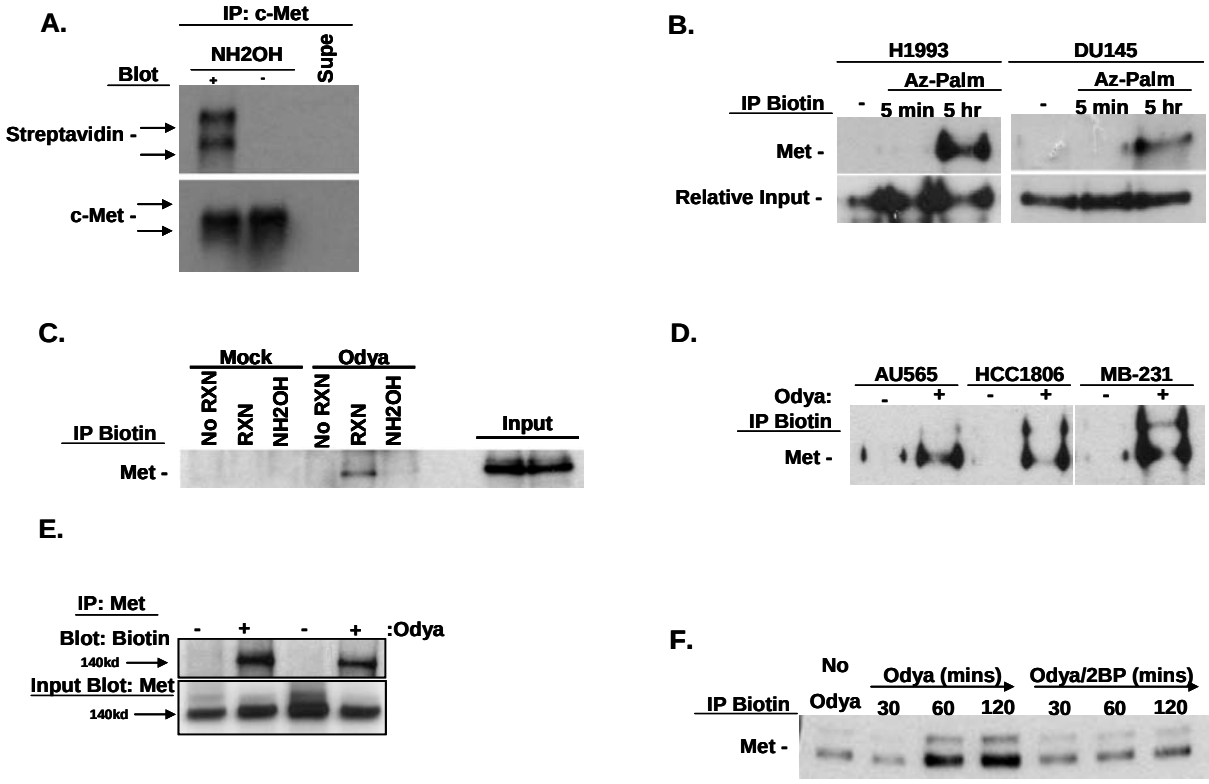


CHAPTER 2 FIGURE LEGEND

Fig. 4. c-Met is palmitoylated via a hydroxylamine-sensitive thioester bond. (A) An acyl-biotin exchange technique involving the blocking of free cysteine residues, removal of palmitate from cysteine by hydroxylamine treatment to expose new free cysteines, and subsequent biotin linkage to these cysteines, was performed on lysates from DU145 cells. Western blot analysis was used to detect biotinylated c-Met. (B) H1993 lung cancer cells or (C) DU145 were incubated with (B) azide- (AZ-Palm) or (C, D) alkyne-linked (Odya) palmitate for the indicated time in order to label palmitoylated proteins. Biotin was subsequently linked to palmitoylated protein via the azide or alkyne moiety following a click chemistry reaction. (C) Palmitate-labeling was performed in DU145 cells to and samples were divided and processed with (Odya) or without (No Odya) palmitate-ortholog labeling, without processing through the click reaction (No RXN), having been reduced prior to the reaction (NH2OH), or without reducing (RXN). (D) Breast cancer cell lines MDA-MB-231, HCC1806, and AU565 were incubated with Odya for 5 hours. Samples were processed through the click reaction to identify palmitoylated protein. For each click reaction experiment, biotinylated protein was subsequently immunoprecipitated and Western blot analysis was performed to detect the presence of c-Met. (E) H1994 cells were incubated with Odya for 5 hours. c-Met was immunoprecipitated and processed through the click reaction. Samples probed for biotin with streptavidin-HRP by Western blotting in order to detect biotinylated c-Met. Two replicate samples are shown along with relative IP input for c-Met. (F) H1993 cells were incubated with Odya in the presence or absence of 100 μM 2BP for 30, 60, or 120 mins.

Biotinylated protein was subsequently immunoprecipitated and western blot analysis was performed to detect the presence of c-Met. Representative blots of triplicate experiments are shown.

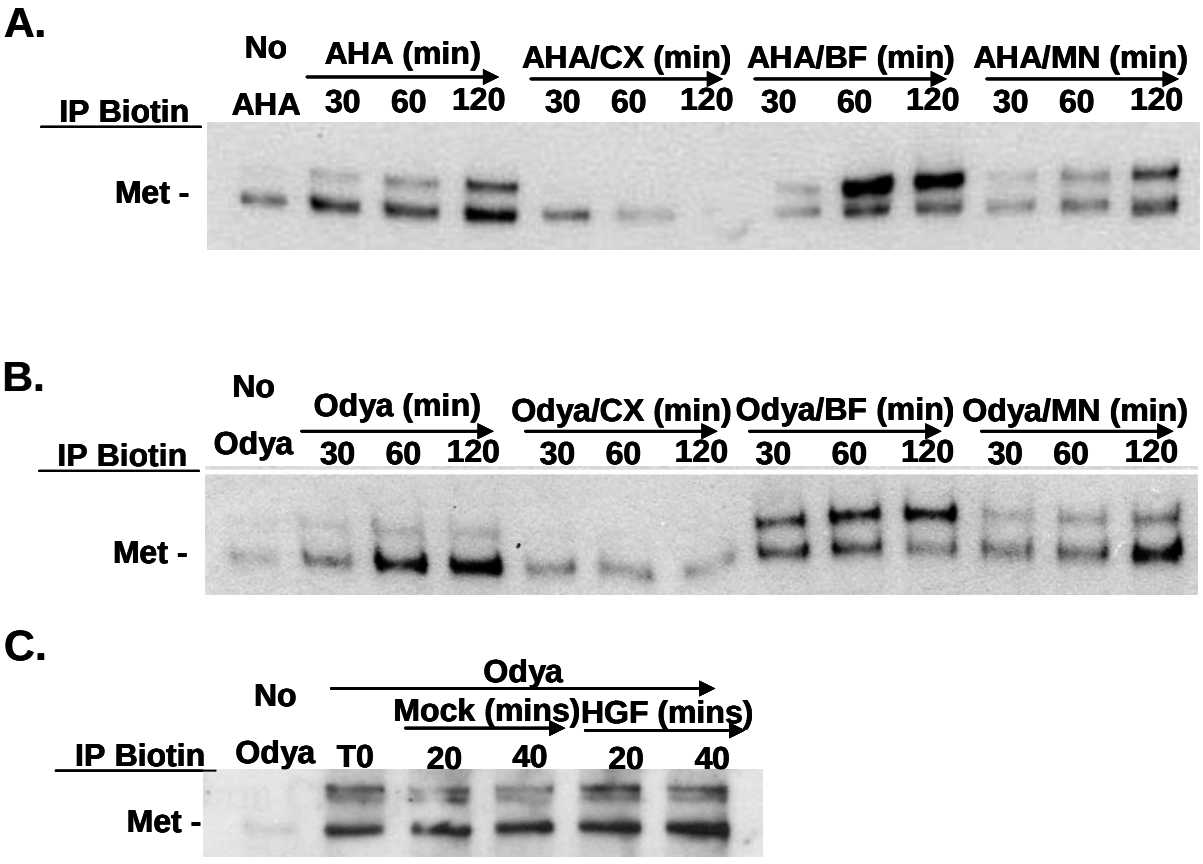
CHAPTER 2 FIGURE



CHAPTER 2 FIGURE LEGEND

Fig. 5. c-Met is stably palmitoylated in the ER. H1993 were incubated with Odya (A) or a methionine-ortholog (AHA) (B) alone or with cycloheximide (CX), brefeldin A (BF), or monensin (MN) for 30, 60, or 120 mins. (C) DU145 cells were incubated with Odya for 2 hours (T0) before chase periods of 20 or 40 minutes in the presence of Odya +/- HGF. For each click reaction experiment, biotinylated protein was subsequently immunoprecipitated and Western blot analysis was performed to detect the presence of c-Met. Representative blots from triplicate experiments are shown.

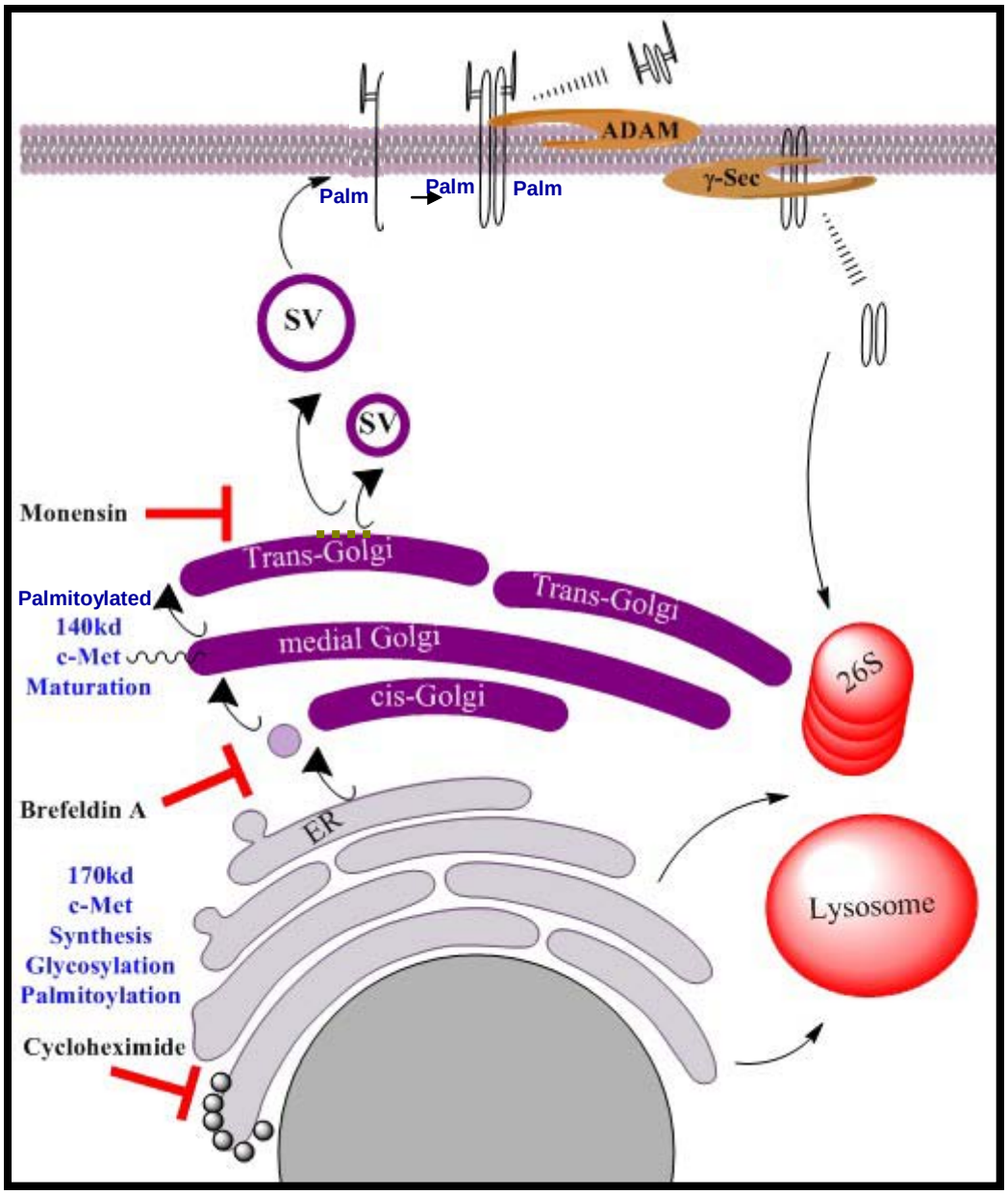
CHAPTER 2 FIGURE



CHAPTER 2 FIGURE LEGEND

Fig. 6. Model for palmitoylation of c-Met. The c-Met protein is synthesized as a 170 kd single chain precursor that is co-translationally glycosylated and cleaved in the Golgi into a disulfide-linked alpha chain (50kd) and beta chain (140kd). The receptor is trafficked through the Golgi to the plasma membrane where it becomes activated by dimerization at a basal rate or increased rate in response to HGF. Upon activation, the receptor is downregulated by proteasomal (26S) or lysosomal degradation. The extracellular domain of c-Met can be cleaved by ADAM (a disintegrin and metalloproteinase) family proteases and shed, leaving the remaining transmembrane fragment to be cleaved by the presenilin-dependent γ-secretase complex (γ-Sec) and subsequently degraded through a proteasomal or lysosomal pathway. Our findings demonstrate that c-Met is palmitoylated within the endoplasmic reticulum (ER) immediately following synthesis, and this is required for eggress from the Golgi and trafficking via secretory vesicles (SV) to the plasma membrane. Cycloheximide treatment was able to prevent incorporation of labeled-palmitate onto c-Met, whereas brefeldin A caused only the precursor form to be palmitoylated and monensin had no effect. Additional evidence suggest that ectodomain shedding may play a role in the loss of c-Met surface expression.

CHAPTER 2 FIGURE

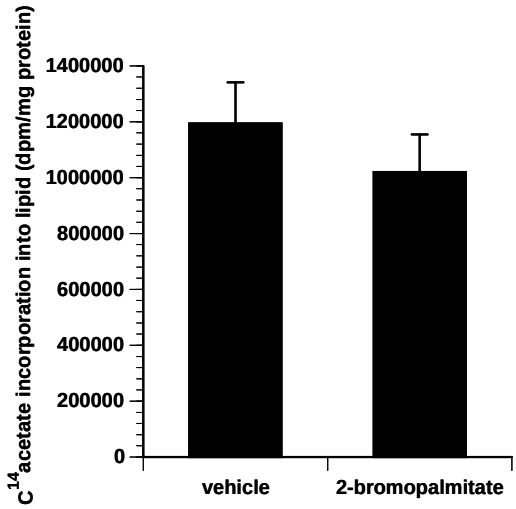


CHAPTER 2 SUPPLEMENTARY FIGURE LEGEND

Supplementary Fig. 1. 2BP treatment does not inhibit FASN activity. (A) DU145 cells were treated with 100 μM 2BP for 2 hours prior to the addition of ^{14}C-acetate for 2 hours. $P<0.05$

CHAPTER 2 SUPPLEMENTARY FIGURE

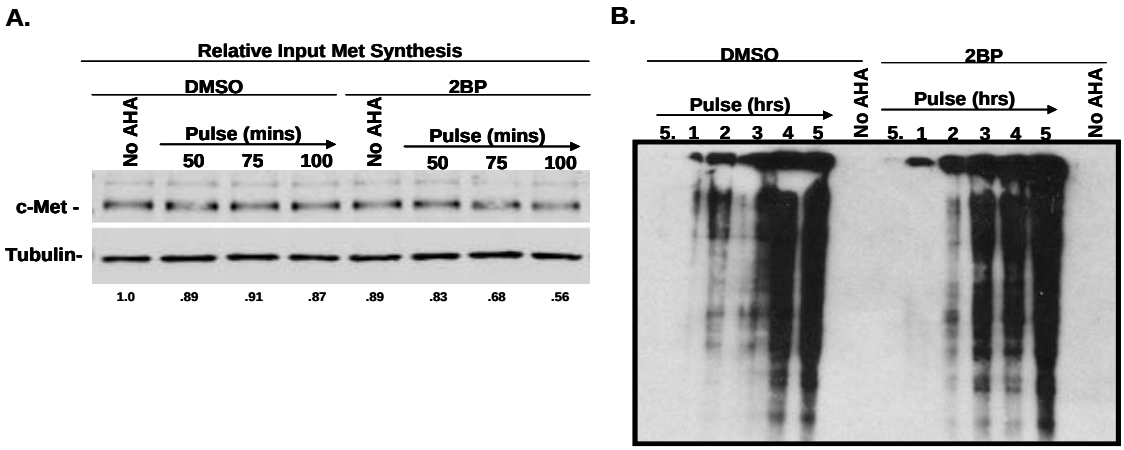
A.



CHAPTER 2 SUPPLEMENTARY FIGURE LEGEND

Supplementary Fig. 2. 2BP treatment does not affect c-Met synthesis. (A) DU145 cells were incubated for 50, 75, or 100 minutes with azido-homoalanine in the presence or absence of 100 μM 2BP. Relative levels of total cell lysate c-Met protein were indicated under each condition by Western blot analysis. (B) DU145 cells were incubated for .5, 1, 2, 3, 4, or 5 hours with azido-homoalanine in the presence or absence of 100 μM 2BP in order to label newly synthesized protein with biotin through a subsequent click chemistry-based reaction. Total cell lysate biotinylated protein was analyzed by Western blotting with Streptavidin-HRP. Representative blots are shown.

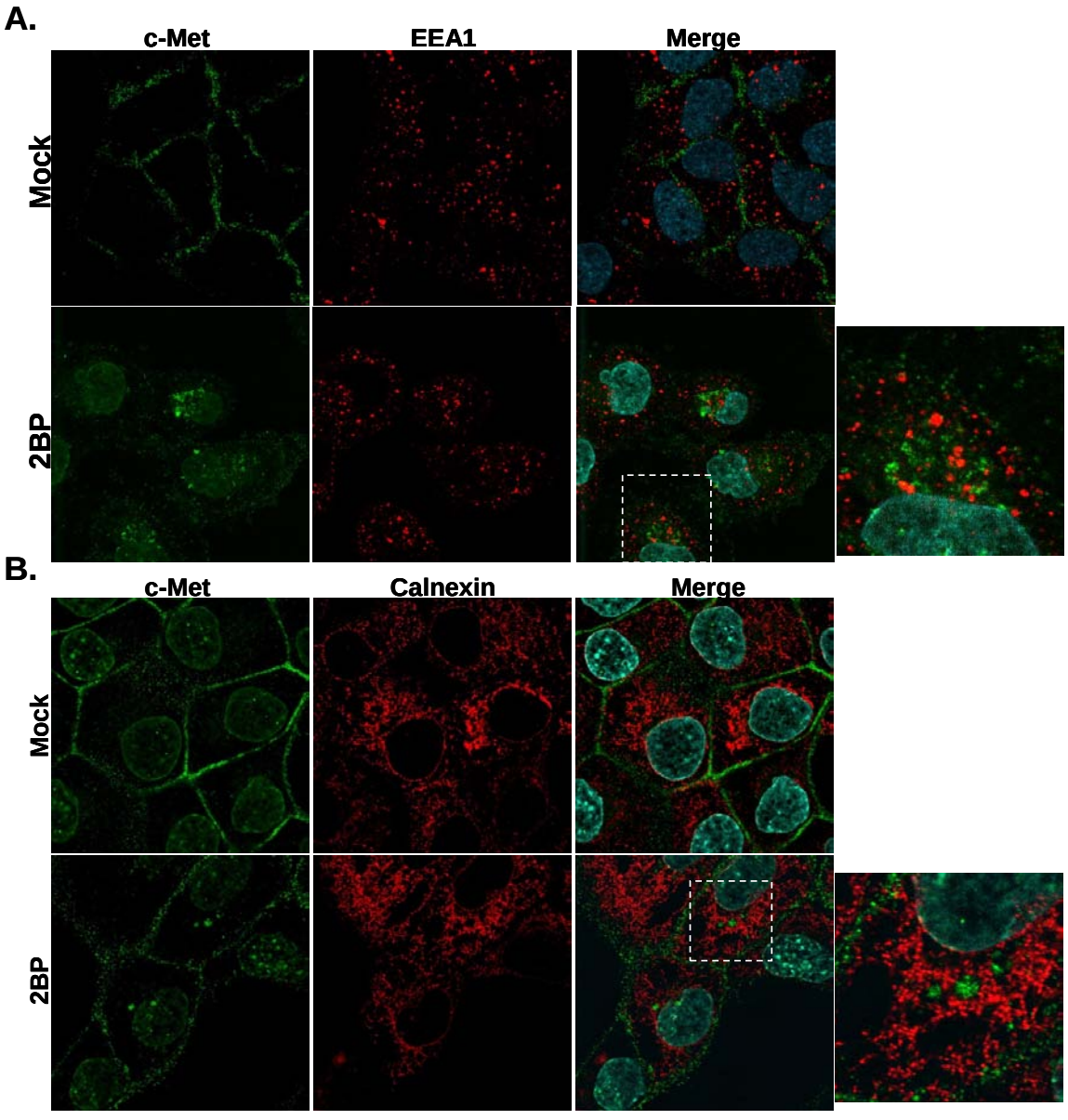
CHAPTER 2 SUPPLEMENTARY FIGURE



CHAPTER 2 SUPPLEMENTARY FIGURE LEGEND

Supplementary Fig. 3. c-Met is does not accumulate in early endosomes or the endoplasmic reticulum during 2BP treatment. DU145 cells were treated with 100 μM 2BP for 2.5 hours prior to fixing. Antibodies to c-Met (green) or the (A) Early Endosome marker EEA1 (red) or (B) ER marker calnexin (red) were used where indicated for immunofluorescence. Representative 60x confocal images are shown.

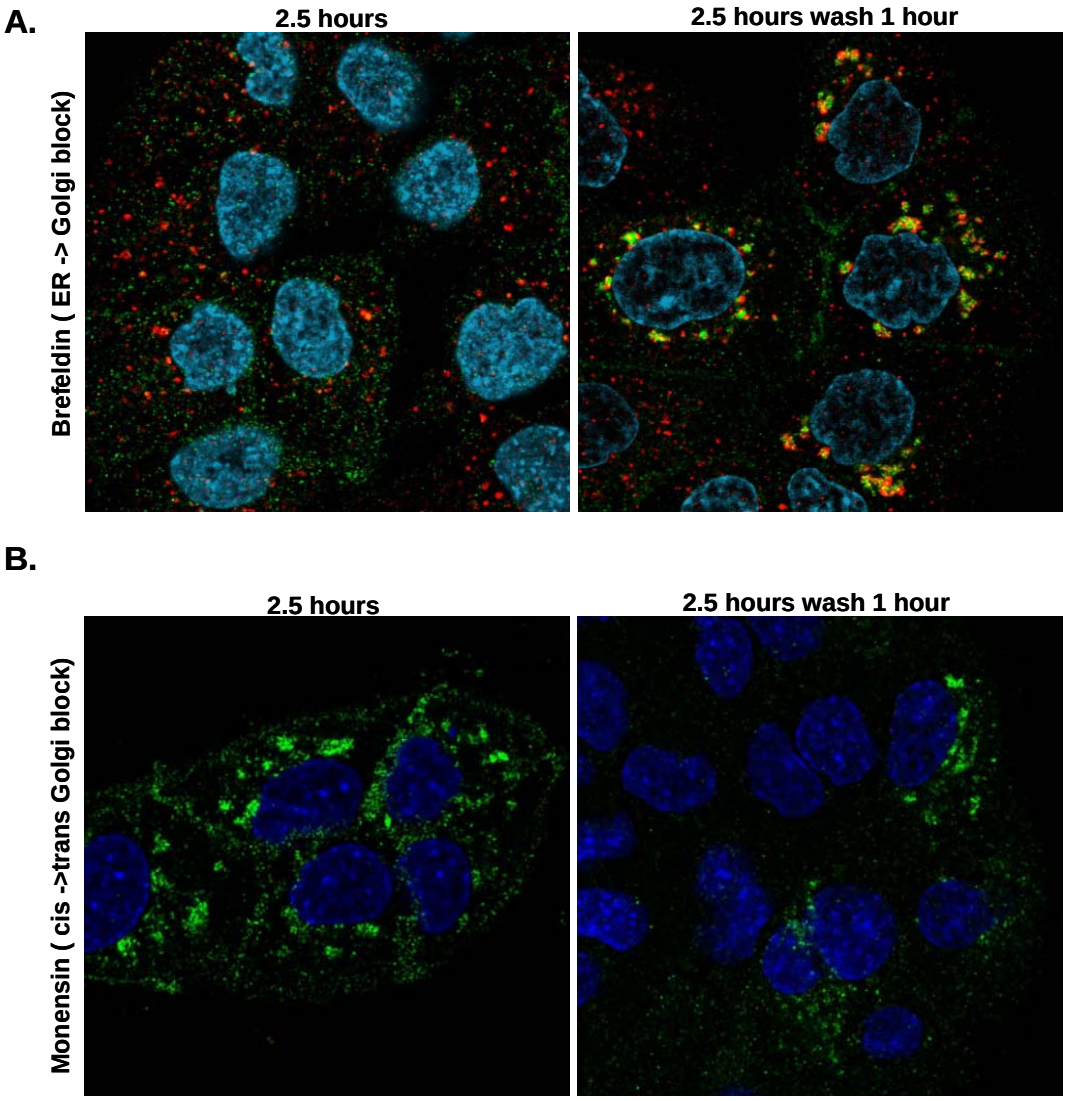
CHAPTER 2 SUPPLEMENTARY FIGURE



CHAPTER 2 SUPPLEMENTARY FIGURE LEGEND

Supplementary Fig. 4. Characterization of brefeldin A and monensin treatment on c-Met subcellular distriubution and proteolytic processing. DU145 cells were treated with (A) a reversible inhibitor of ER eggress, BF, at 1 μg/ml or a reversible inhibitor of cis- to trans-Golgi trafficking, MN, for 2.5 hours prior to fixing. In addition, cells were treated and followed with drug removal by washing and an additional 2.5 hour chase period. The antibody used for c-Met (green) detection by IF was not able to detect precursor c-Met, but following the removal of BF, the mature form is detected emerging in the cis-Golgi (red) having been processed. Representative 60x confocal images are shown.

CHAPTER 2 SUPPLEMENTARY FIGURE



CHAPTER 2 SUPPLEMENTARY FIGURE LEGEND

Supplementary Table 1. List of strategies tested for ability to prevent downregulation of c-Met in response to FASN or palmitoylation inhibition. DU145 cells were treated with 2-BP 3 or C75 for 3 and 6 hours in the presence or absence of the indicated drugs. In addition, shRNA knockdown of proteins critical for particular internalization mecanisms was used in the presence or absence of 2BP or C75. None of the listed strategies prevented c-Met loss by greater than 10%.

CHAPTER 2 SUPPLEMENTARY FIGURE

Supplementary Table 1.

Drug	Target
Chlorpromazine	Clathrin Pit Formation
MG132	proteosome
Concanamycin A	lysosome acidification
Okadaic Acid	Ser phosphatase
LY294002	PI3K
C3 Transferase	RhoA,B,C
Vinblastine	microtubules
PP2	src family
Y27632	Rock1-II
UO126	MEK1 and 2
BIM-1	PKC
Latrunculin	actin
SB203580	p38, pdk1
SP600125	JNK
Dansylcadaverine	Clathrin pit formation
Chloroquine	lysosome acidification
bafilomycin	V-ATPase, lysosomal acidification, endosomal pH
bpV(phen)	Y phosphatase
Z-VAD-FMK	caspases
EIPA	Na/H Exchangers, endosomal pH gradients

Drug	Target
Hyperosmotic Sucrose	Clathrin pit formation
PAO	Y Phosphatase, Endocytosis
Cytochalasin D	Depolymerizes Actin
Rottlerin	Fluid Phase Endocytosis
Amphotericin B	Lipid Rafts (intercalates within cholesterol rich domains)
Dynasore	Dynamin
mBCD	Lipid Rafts (chelates/extracts cholesterol)
Nystatin	Lipid Rafts (binds cholesterol)
Nocodazole	Depolymerizes microtubules
CHC -/-	Clathrin pits
Dyn -/-	Dynamin dependent endocytosis
RhoA -/-	Subset of endocytosis mechanisms

References:

1. Trusolino, L., A. Bertotti, and P.M. Comoglio, MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol, 2010. **11**(12): p. 834-48.
2. Giordano, S., et al., Biosynthesis of the protein encoded by the c-met proto-oncogene. Oncogene, 1989. **4**(11): p. 1383-8.
3. Park, M., et al., Mechanism of met oncogene activation. Cell, 1986. **45**(6): p. 895-904.
4. Lefebvre, J., et al., Met degradation: more than one stone to shoot a receptor down. Faseb J, 2012. **26**(4): p. 1387-99.
5. Abella, J.V., et al., Dorsal ruffle microdomains potentiate Met receptor tyrosine kinase signaling and down-regulation. J Biol Chem, 2011. **285**(32): p. 24956-67.
6. Parachoniak, C.A., et al., GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. Dev Cell, 2012. **20**(6): p. 751-63.
7. Ancot, F., et al., Shedding-generated Met receptor fragments can be routed to either the proteasomal or the lysosomal degradation pathway. Traffic, 2012. **13**(9): p. 1261-72.
8. Nath, D., et al., Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. J Cell Sci, 2001. **114**(6): p. 1213-1220.
9. Petrelli, A., et al., Ab-induced ectodomain shedding mediates hepatocyte growth factor receptor down-regulation and hampers biological activity. Proc Natl Acad Sci U S A, 2006. **103**(13): p. 5090-5.
10. Chaudhuri, A., et al., Distinct involvement of the Gab1 and Grb2 adaptor proteins in signal transduction by the related receptor tyrosine kinases RON and MET. J Biol Chem, 2011. **286**(37): p. 32762-74.

11. Weidner, K.M., et al., Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J Cell Biol, 1990. **111**(5 Pt 1): p. 2097-108.

12. Stoker, M., et al., Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature, 1987. **327**(6119): p. 239-42.

13. Ma, P.C., et al., c-Met: Structure, functions and potential for therapeutic inhibition. Cancer and Metastasis Reviews, 2003. **22**(4): p. 309-325.

14. Peters, S. and A.A. Adjei, MET: a promising anticancer therapeutic target. Nat Rev Clin Oncol, 2012. **9**(6): p. 314-26.

15. Wickramasinghe, D. and M. Kong-Beltran, Met activation and receptor dimerization in cancer: a role for the Sema domain. Cell Cycle, 2005. **4**(5): p. 683-5.

16. Boccaccio, C., et al., Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature, 1998. **391**(6664): p. 285-8.

17. Kermorgant, S. and P.J. Parker, Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol, 2008. **182**(5): p. 855-63.

18. Kermorgant, S. and P.J. Parker, c-Met signalling: spatio-temporal decisions. Cell Cycle, 2005. **4**(3): p. 352-5.

19. Gherardi, E., et al., Targeting MET in cancer: rationale and progress. Nat Rev Cancer, 2012. **12**(2): p. 89-103.

20. Engelman, J.A., et al., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science, 2007. **316**(5827): p. 1039-43.

21. Rho, J.K., et al., The role of MET activation in determining the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. Mol Cancer Res, 2009. **7**(10): p. 1736-43.

22. Ogino, S., et al., Cohort study of fatty acid synthase expression and patient survival in colon cancer. J Clin Oncol, 2008. **26**(35): p. 5713-20.

23. Turke, A.B., et al., Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell, 2012. **17**(1): p. 77-88.

24. Wilson, T.R., et al., Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. 2012. advance online publication.

25. Christensen, J.G., J. Burrows, and R. Salgia, c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett, 2005. **225**(1): p. 1-26.

26. Catenacci, D.V., et al., Durable complete response of metastatic gastric cancer with anti-Met therapy followed by resistance at recurrence. Cancer Discov, 2012. **1**(7): p. 573-9.

27. Steffan, J.J., D.T. Coleman, and J.A. Cardelli, The HGF-met signaling axis: emerging themes and targets of inhibition. Curr Protein Pept Sci, 2011. **12**(1): p. 12-22.

28. Coleman, D.T., R. Bigelow, and J.A. Cardelli, Inhibition of fatty acid synthase by luteolin post-transcriptionally down-regulates c-Met expression independent of proteosomal/lysosomal degradation. Mol Cancer Ther, 2009. **8**(1): p. 214-24.

29. Uddin, S., et al., Inhibition of fatty acid synthase suppresses c-Met receptor kinase and induces apoptosis in diffuse large B-cell lymphoma. Mol Cancer Ther, 2010. **9**(5): p. 1244-55.

30. Medes, G., A. Thomas, and S. Weinhouse, Metabolism of Neoplastic Tissue. IV. A Study of Lipid Synthesis in Neoplastic Tissue Slices in Vitro. Cancer Res, 1953. **13**(1): p. 27-29.

31. Menendez, J.A., et al., Does endogenous fatty acid metabolism allow cancer cells to sense hypoxia and mediate hypoxic vasodilatation? Characterization of a novel molecular connection between fatty acid synthase (FAS) and hypoxia-inducible factor-1alpha (HIF-1alpha)-related expression of vascular endothelial growth factor (VEGF) in cancer cells overexpressing her-2/neu oncogene. J Cell Biochem, 2005. **94**(5): p. 857-63.

32. Rysman, E., et al., De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Res, 2010. **70**(20): p. 8117-26.

33. Kuhajda, F.P., Fatty Acid Synthase and Cancer: New Application of an Old Pathway. Cancer Res, 2006. **66**(12): p. 5977-5980.

34. Swinnen, J.V., et al., Overexpression of fatty acid synthase is an early and common event in the development of prostate cancer. International Journal of Cancer, 2002. **98**(1): p. 19-22.

35. Swinnen, J.V., et al., Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochemical and Biophysical Research Communications, 2003. **302**(4): p. 898-903.

36. Vazquez-Martin, A., et al., Pharmacological blockade of fatty acid synthase (FASN) reverses acquired autoresistance to trastuzumab (Herceptin by transcriptionally inhibiting 'HER2 super-expression' occurring in high-dose trastuzumab-conditioned SKBR3/Tzb100 breast cancer cells. Int J Oncol, 2007. **31**(4): p. 769-76.

37. Fiorentino, M., et al., Overexpression of fatty acid synthase is associated with palmitoylation of Wnt1 and cytoplasmic stabilization of beta-catenin in prostate cancer. Lab Invest, 2008. **88**(12): p. 1340-8.

38. Wei, X., et al., Fatty acid synthase modulates intestinal barrier function through palmitoylation of mucin 2. Cell Host Microbe, 2012. **11**(2): p. 140-52.

39. Wei, X., et al., De novo lipogenesis maintains vascular homeostasis through endothelial nitric-oxide synthase (eNOS) palmitoylation. J Biol Chem, 2011. **286**(4): p. 2933-45.

40. Triola, G., H. Waldmann, and C. Hedberg, Chemical biology of lipidated proteins. ACS Chem Biol, 2011. **7**(1): p. 87-99.

41. Resh, M.D., Trafficking and signaling by fatty-acylated and prenylated proteins. Nat Chem Biol, 2006. **2**(11): p. 584-90.

42. Navarro-Lerida, I., et al., A palmitoylation switch mechanism regulates Rac1 function and membrane organization. Embo J, 2011. **31**(3): p. 534-51.

43. Lee, H., et al., Palmitoylation of caveolin-1 at a single site (Cys-156) controls its coupling to the c-Src tyrosine kinase: targeting of dually acylated molecules (GPI-linked, transmembrane, or cytoplasmic) to caveolae effectively uncouples c-Src and caveolin-1 (TYR-14). J Biol Chem, 2001. **276**(37): p. 35150-8.

44. Roberts, P.J., et al., Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. J Biol Chem, 2008. **283**(37): p. 25150-63.

45. La Rosa, P., et al., Palmitoylation regulates 17beta-estradiol-induced estrogen receptor-alpha degradation and transcriptional activity. Mol Endocrinol, 2012. **26**(5): p. 762-74.

46. Drisdel, R.C., et al., Assays of protein palmitoylation. Methods
Protein Palmitoylation, 2006. **40**(2): p. 127-134.

47. Draper, J.M. and C.D. Smith, Palmitoyl acyltransferase assays and inhibitors (Review). Mol Membr Biol, 2009. **26**(1): p. 5-13.

48. Jennings, B.C., et al., 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. J Lipid Res, 2009. **50**(2): p. 233-42.

49. Resh, M., Use of analogs and inhibitors to study the functional significance of protein palmitoylation. Methods, 2006. **40**(2): p. 191-7.

50. Webb, Y., L. Hermida-Matsumoto, and M.D. Resh, Inhibition of Protein Palmitoylation, Raft Localization, and T Cell Signaling by 2-Bromopalmitate and Polyunsaturated Fatty Acids. Journal of Biological Chemistry, 2000. **275**(1): p. 261-270.

51. Gagnoux-Palacios, L., et al., Compartmentalization of integrin alpha6beta4 signaling in lipid rafts. J Cell Biol, 2003. **162**(7): p. 1189-96.

52. Yang, X., et al., Palmitoylation supports assembly and function of integrin-tetraspanin complexes. J Cell Biol, 2004. **167**(6): p. 1231-40.

53. Yang, Y.Y., J.M. Ascano, and H.C. Hang, Bioorthogonal chemical reporters for monitoring protein acetylation. J Am Chem Soc, 2010. **132**(11): p. 3640-1.

54. Surma, M.A., C. Klose, and K. Simons, Lipid-dependent protein sorting at the trans-Golgi network. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids

Lipids and Vesicular Transport, 2012. **1821**(8): p. 1059-1067.

55. Klemm, R.W., et al., Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-Golgi network. The Journal of Cell Biology 2009 **185** (4): p. 601-612.

SIGNIFICANCE, CONCLUSIONS, AND FUTURE CONSIDERATIONS

The current paradigm for targeted therapeutic design is focused on inhibiting the activity of known oncogenic kinases. Although this strategy has provided several successful drugs, a few consistent overarching hindrances have become apparent. First, although it's a more direct target-to-outcome strategy, targeting kinase activity can have limited effect. Using small molecules or monoclonal antibodies to obstruct ATP-binding sites or ligand binding is a popular strategy, but it is molecularly simpler to overcome. Point mutations, heterodimerization, and overexpression have all been demonstrated as mechanism to resist targeted cancer therapeutics. An alternative strategy focused on preventing the expression of the protein of detriment may be a more efficient approach. In addition, this strategy may more easily allow for the targeting of proteins within a related cancer profile en masse, rather than limiting the effect to an individual target.

This concept introduces a second challenge for clinical progress. At its very nature, cancer is an unstable, ever-changing, and; therefore, heterogeneous disease. While there are certain examples of more clonal disease that are driven so aggressively by one aberration that the patient dies before heterogeneous populations develop, the majority of cancers accumulate oncogenes and lose suppressors with the passage of time resulting in an immensely difficult clinical presentation to tackle. In fact, the highly aggressive, but more clonal cancers currently have exceptional survival rates due to development of targeted agents, and consistently exhibit lower rates of relapse. The greatly improved survival of patients with chronic myelogenous leukemia driven by the BCR-ABL fusion is a perfect example. With this caveat, the majority of cancers are not being effectively treated by a single target approach. More and more it is found that

either prior to the initiation of treatment or actually induced by treatment, compensatory pathways arise that circumvent the intended activity of therapy. Albeit, the bulk of tumor cells are often oncogenically "addicted" to the target of therapy and will initially show dramatic response to treatment. However, with time, the cancer recurs due to the outgrowth of resistant cells driven by an alternative mitogenic pathway, emphasizing the necessity for multimodality approaches designed with oncogenic profiles such as the lipogenic phenotype in mind.

The third major hindrance relates more directly to the aforementioned resistant outgrowth. In recent years, a large body of data has identified a unique subset of cancer cells within most tumors that remain dormant and; thus, biochemically masked to most anti-mitotic therapeutic agents. This population seems to represent the foundation from which cancer relapse occurs. The cells exhibit striking similarities to stem-cells. They are characteristically resistant to most forms of therapy including chemotherapy and radiation therapy. It is not currently understood whether this population is present at the dawn of the tumor or whether they arise in response to intratumoral environmental stresses and/or treatment. What does seem to hold consensus, is that the c-Met signaling pathway is a major contributor to the development and maintenance of these dedifferentiated stem-like cells. To have greater success with future treatment strategies, these dormant cells must be eliminated along with the bulk of the tumor.

Taken together, the introduction of these concepts serves to emphasize the importance of building on past successes, by addressing the prevailing limitations, rather than futilely repeating precedence. The data acquired for this body of work was done so keeping these up-to-date overarching clinical challenges in focus. To address these

limitations, it is essential to focus on targets that are dominant at the most pivotal stages of disease. Important targets for therapeutic oncology are those that control bulk tumor growth; but more importantly those that control the lethal invasive spread as well as the weakly-mitotic, yet treatment-resistant, cancer stem-like population. Based on information provided at the beginning of this document, it is the belief of this author that the receptor tyrosine kinase c-Met meets these criteria. One should consider novel means of repressing the target, including downgrading its stability or expression in order to limit possibilities of acquired resistance. To this end, it is advantageous to find broadly influential, but cancer selective, cellular components and processes with the potential of restricting a common proteomic profile, not just a single target protein. Investigation into the phenotypes, such as the lipogenic phenotype, that allow for a target protein's expression is warranted based on this theory. This stance increases the potential for multimodality value, particularly as more common cancer regulatory and/or causational themes are discovered. The results presented here fit within this model for addressing current challenges and are a solid foundation for extensive future studies.

Discussed in this thesis were findings acknowledging the interconnectedness of a pleiotropic growth factor receptor, a lipid modification known to influence protein stability and trafficking, and a metabolic enzyme associated with an oncogenic profile that can provide lipids for this modification. The growth factor receptor c-Met is essential for formation and repair of normal tissue architecture. Signaling through this receptor induces changes to downstream protein activity and gene expression adjustments that culminate in context-dependent phenotypes related to the rapid proliferation of cells or remodeling of tissue structure. However, as described, when there is a breakdown of

the control mechanisms for expression and/or downregulation, the overabundant signal causes adverse effects - principally cancer and its progression to invasive disease. There is plentiful data, from cell culture, animal models, and clinical analysis, that c-Met is one of the most common and potent proteins dysregulated in cancer. Based on the receptor's normal physiological responsibility as a mediator of rapid tissue repopulation and invasive outgrowth and migration, it is relatively simple to envision the results of unabated c-Met signaling as it relates to cancerous growth and spread.

Fatty acid synthesis is a unique metabolic process that is utilized only under select physiological conditions, such embryonic development, lactating breast duct formation, and almost universally in oncogenesis. If its role is truly necessary for cancer cell survival and/or spread, as most evidence suggests, the common abnormal expression of FASN in cancer cells makes it a highly attractive therapeutic target. Moreover, the lipid products from FASN activity influence a multitude of cellular functions. Although it is not fully accepted why cancer cells need FASN, explanations including phospholipid biosynthesis, protein acylation, redox maintenance, and energy storage have all been proposed and defended with some evidence. Given the common upregulation of FASN expression in cancer and the multitude of cellular process that could be affected by its activity, it is reasonable to assume that proteins could be incongruously affected by a hyperactive lipogenic phenotype either allowing them or causing them to be oncogenic. This idea will be elaborated later in this text.

Providing lipid for the acylation of proteins is one of the proposed responsibilities for FASN that contributes to oncogenesis. Acylation regulates many cancer-associated proteins, in general, by adjusting their hydrophobicity and; therefore, membrane

association and spatial regulation. However, from numerous studies finding exceedingly diverse roles for seemingly similar acylation patterns on different proteins, it would seem there are excessive subtleties introduced by this protein modification. As was thoroughly discussed, dynamic palmitoylation has been shown to control particular proteins' exit from and recycling to different organelles. It is also required for the proper conformational folding of some proteins; and thus, necessary for their maintained stability. An important question that remains in this field is how the process of acylation is regulated and if there are disparities in the context of cancer. Speculations on this question will also be presented shortly. Nevertheless, the data presented in this thesis established that *de novo* synthesized palmitate from FASN is necessary for the palmitoylation of c-Met, and this palmitoylation is required for c-Met to exit the Golgi and reach the plasma membrane.

Our initial efforts sought to indentify compounds that downregulated c-Met signaling by unique means, in the expectation of providing novel therapeutic strategies to the clinic. Screening a class of compounds known as flavonoids, we identified luteolin as a promising hit. We subsequently demonstrated that luteolin lowered total c-Met protein levels subsequent to transcription, and this, as expected, coincided with the reduced sensitivity of cancer cell lines to HGF stimulation. c-Met protein synthesis was also determined not to be affected, suggesting it was being degraded at an increased rate. However, experiments testing this hypothesis with respect to the proteasome and lysosome, suggested an unknown alternative path for c-Met loss. Building on previous reports that luteolin was a potent inhibitor of FASN, we found that specific FASN

inhibition or shRNA knockdown could lower c-Met protein levels, and importantly, the addition of exogenous palmitate could prevent this effect.

Palmitate is the predominant end-product of FASN activity, and can be utilized by the cell for diverse needs including post-translational modifications. We tested the idea that reduced intracellular palmitate caused by FASN inhibition was acting to reduce cellular palmitoylation to lower c-Met levels. A palmitoylation inhibitor, but not inhibitors to other known lipid modifications, was able to lower c-Met similar to FASN inhibition. Also paralleling FASN inhibition, 2BP treatment was found not to effect the transcription or translation of c-Met, but rather it appears to reduce the stability of newly synthesized protein. Analysis of the subcellular distribution of c-Met revealed that inhibition of palmitoylation caused a pool of c-Met to accumulate in the Golgi and to be diminished from the plasma membrane over time.

These results hinted at two possibilities. Either c-Met was palmitoylated, and this was required to maintain its stability; or other chaperone-like proteins necessary for c-Met conformation and stability require palmitoylation for proper function. Two distinct techniques were applied to confirm that c-Met was actually palmitoylated in all cell lines tested. Furthermore, the kinetics of palmitoylation were investigated using inhibitors of distinct steps of the biosynthetic and secretory pathway. We found that c-Met is palmitoylated within the ER in its precursor form, and this modification is necessary for egress from the Golgi along the secretory pathway to the plasma membrane.

It is possible that c-Met requires palmitoylation to sequester within Golgi-associated lipid rafts for delivery to the plasma membrane. Unlike some other palmitoylated proteins, we did not detect dynamic turnover (palmitoylation or

depalmitoylation) of the modification under basal or ligand-stimulated conditions. The palmitoylation of an oncogenic receptor tyrosine kinase is unprecedented. Because of the great clinical importance of c-Met, these findings have the potential to reinvigorate interest in the development of acylation inhibitors for therapeutic use.

These results are of high impact for providing supporting evidence of a potentially common biosynthesis regulatory mechanism, for adding c-Met to the repertoire of cellular components influenced by lipogenesis, for specifically adding to the understanding of c-Met regulation, and for identifying a unique means of targeting a clinically-relevant receptor tyrosine kinase. In addition, some of the work demonstrated here was acquired using state-of-the art techniques with limited precedence that could be adopted to expedite the future of palmitoylation research. These findings establish a foundation that could be built upon to address many of the overarching challenges currently frustrating the field of clinical oncology by focusing on the lipogenic profile and associated acylation to stop invasive and resistant phenotypes exemplified by c-Met.

In the short-term, there are a number of questions that would add great weight to the data presented in this thesis. Much of the future progression of this project hinges on the identification of the one or more palmitoylated cysteine residue/s on c-Met. Mass spectrometry and site-directed mutagenesis techniques will continue to be applied for this purpose, and once identified, palmitoylation-defective c-Met mutants can be used for future experimentation. A major limitation to the work thus-far has been the inability to specifically block palmitoylation of c-Met, rather than preventing global palmitoylation with 2BP. We were unable to demonstrate definitive effects of preventing c-Met palmitoylation at the cellular level because results of 2BP treatment could be attributable

to any number of other palmitoylated proteins. GFP-tagged c-Met mutants will allow us to determine whether palmitoylation of c-Met is, in fact, required for any amount of expression in cell lines, and whether this holds true in 3D *in vitro* models and xenograft animal models of c-Met driven cancer. Moreover, site-specific genome editing in mice could be used to elucidate the role of c-Met palmitoylation during embryonic development and in numerous transgenic mouse models of cancer initiation and progression. Knowing that c-Met expression is required for embryonic development, we would predict embryonic lethality with palmitoylation mutants necessitating an inducible system for studies in adult tissue.

More firmly establishing the connection from FASN, to acylation, to c-Met, is an additional important goal to address. Our data, demonstrate that the end product of FASN activity, palmitate, is a necessary element required for c-Met stability and that palmitate must be attached to c-Met for maintained stability; however, we have not directly shown, although it is reasonable to predict, FASN-derived palmitate is selectively utilized for such palmitoylation. This experiment can be done by metabolically labeling with ^{14}C-acetate which will be incorporated as substrate into palmitate synthesis. It would then be possible to test if FASN inhibitors block palmitoylation of c-Met. Other laboratories have done this on occasion to demonstrate FASN-dependent palmitoylation, but to definitively bridge our results on FASN inhibition and palmitoylation it would be beneficial to confirm under our experimental conditions. Results from analysis of tissue microarrays examining the expression of FASN and c-Met and different types and stages of cancer will be highly informative toward establishing this connection.

Our data would lead one to propose that FASN is required for c-Met expression, but to what extent this holds true is unknown. How much FASN would be enough? Do levels directly correlate, or are low levels of FASN adequate to support highly elevated c-Met levels? Does c-Met "prefer" *de novo* palmitate, but exogenous sources are sufficiently available *in vivo*? Are adequate exogenous sources only available under particular conditions in cancer, therein explaining the need for FASN? This same question could be extended to FASN and c-Met expression during embryonic development and tissue repair. Extensive analysis of expression patterns in histological samples could help address many of these fundamental questions, and could provide suggestive evidence to defend or refute FASN-dependent palmitoylation of c-Met *in vivo*. Furthermore, testing the ability of FASN inhibitors to lower c-Met expression in xenograft animal models could establish proof-of-principle data supporting the therapeutic potential of this connection.

Our data consistently demonstrate a significant net loss of total c-Met protein levels in response to both FASN inhibition and inhibition of palmitoylation; however, the precise mechanism responsible for degradation or loss from the cell has remained elusive. We have performed numerous experiments using inhibitors to the common degradation pathways, as discussed in the previous chapters, but either compensatory regulatory mechanisms or indirect effects of inhibition were masking the results or there is truly no role for these pathways. It did; however, appear that, at least in part, plasma membrane associated c-Met was shed from the cell in response to FASN or palmitoylation inhibition. Several follow-up studies will need to be performed to demonstrate conclusively the protease responsible for this cleavage, the initiating signal cascade, and

if there is any unique cellular consequence to this shedding event. Again, although this is caused by inhibition of FASN or palmitoylation, there is no evidence that this is due to preventing palmitoylation of c-Met specifically. Because we did not see palmitoylation turnover beyond exit from the Golgi, it is unlikely that depalmitoylation of c-Met at the plasma membrane would have any normal regulatory function. Why inhibition of FASN or palmitoylation results in this shedding is still an important question to address.

The mechanism by which accumulated unpalmitoylated c-Met in the Golgi is eliminated also remains to be determined. There are a multitude of oligopeptidases resident to the ER and Golgi that are potentially responsible and can be systematically tested using commercially available inhibitors; however, thus far we have not established a conclusion. If this strategy fails, it would seem likely that the accumulated pool in the Golgi is slowly degraded via a lysosome or proteasome mechanism, but when inhibitors were used to test this hypothesis, they caused a more rapid degradation of the plasma membrane pool, thereby appearing as if there is no difference in c-Met loss. A definitive conclusion for this question could possibly be determined with a series of experiments meticulously addressing each subtle possibility.

During the course of the work presented herein, a number of equally important and exceedingly intriguing conjectures have arisen. A greater understanding of the biosynthesis, trafficking, and stability of c-Met is of great importance, particularly for the field of cancer; but this research has brought up some broader ideas with respect to the role FASN and acylation play in cell biology. Prior to the identification of a class of enzymes that could catalyze palmitoylation, PATs, it was proposed that the process may be a spontaneous occurrence, termed autoacylation. Little actual evidence or

hypothetical elaborations were ever presented, but this author feels that, under certain circumstances, the possibility may remain. Overabundant and hyperactive FASN, given sufficient substrate, could yield an elevated concentrate of intracellular palmitate. Given the law of mass action, palmitate would more likely be "activated" to palmitoyl-CoA. Even if restrictive mechanisms were in place they could potentially be superseded. The abnormally high levels of palmitoyl-CoA could spontaneously form thioester linkage with available cysteine residues. As mentioned in the literature review, there is evidence that ectopic overexpression of FASN alone can result in global changes in protein trafficking, which ultimately disrupted normal cell polarity (77). In this case, it is possible that the heightened FASN levels caused an aberrant over-acylation of cellular proteins resulting in spatial disarray.

One could speculate that this is a common driving force for cancer initiation and exacerbation. If this speculation were true, one would predict an almost random palmitoylation-site selection on diverse proteins in cells with overexpression of FASN. This model could easily be tested through ectopic expression of FASN in cell lines and analysis of protein palmitoylation; however, a more likely possibility is less extreme. Excess palmitate could still bias the palmitoylation of proteins, but it may remain a controlled, enzyme-catalyzed reaction at designated residues. This hypothesis would be more in agreement with the study demonstrating FASN overexpression increases the levels of palmitoylated Wnt-1 in both cancer cells and cancer tissue samples.

Returning to c-Met, it has been mentioned that the receptor has limited expression in most tissues, besides being expressed in cancers or in normal adult or embryonic tissues with stem-like characteristics. In general, this is the same pattern of expression

observed with FASN. During embryonic development, FASN was elevated in regions of dynamic turnover and remodeling, similar to c-Met. FASN is expressed in lactating breast tissue, and important for the tubulogenesis of lactating breast ducts. Tissues regions that share high FASN and c-Met, tend to exhibit high cellular activity and a dedifferentiated phenotype, similarly observed in cancer. One could theorize that the lipogenic phenotype, characterized by elevated FASN, acts as a master regulator of this phenotype. Abundant FASN-derived palmitate could be the activation signal allowing dynamically palmitoylated proteins to be regulated differently, and stably palmitoylated proteins, such as proposed for c-Met, to be expressed at all or to higher levels. Preliminary data from our laboratory does suggest that ectopic overexpression of FASN elevates the expression of c-Met, but there is a level of context-dependence that requires additional investigation.

The concept of targeting protein acylation for therapeutic benefit has been mitigated by the worry of excess toxicity due to its general requirement in normal physiology. However, this apprehension may not be universally warranted. First, although there is currently limited data to support this, it is reasonable to assume that a.) acyltransferases are aberrantly expressed and/or active in diseased tissue and b.) cancer cells may be "addicted" to the activity of palmitoylated oncogenes thereby making cancer cells more sensitive to the inhibition of acyltransferases. Second, the plethora of palmitoyl-acyltransferases suggests a much greater level of specificity for their cognate palmitoylated protein. Compared to isoprenylation and myristoylation of diverse proteins catalyzed by single enzymes, palmitoylation exhibits much greater individuality from the dozens of known PATs. Therefore, targeting specific PAT-palmitoylated protein pairings

could be done with greater accuracy and limited off-target involvement. Taken further, it may be found that particular PATs are expressed as part of a particular disease-associated phenotypic profile increasing their potential as biomarkers and therapeutic targets, as well as linking their known palmitoylated protein substrates with the disease.

In conclusion, this thesis presents the foundation for a field of investigation into the role of elevated lipogenesis in normal and diseased cell biology through its influence on protein acylation. This foundation developed out of the findings that, under the conditions tested, c-Met required FASN activity and ultimately palmitoylation in order to be properly expressed at the cell surface. Establishing the requirement of fatty acid regulation with a receptor essential for several unique cellular phenotypes in normal and diseased states advanced a broader hypothesis. Although there is extensive literature documenting the regulation and effects of lipogenesis and acylation, there is a void in connecting the two as causative agents that drive cell phenotypes. More frequently the two are discussed separately and/or as byproducts of a phenotypic change - being regulated by the change rather than causing the change. Defending this hypothesis could have great clinical impact and should be tested in future work.

APPENDIX I

Abbreviations

ABBREVIATIONS

β4	Integrin β4
γ-Sec	Presenilin-Dependent γ-Secretase Complex
2-BP	2-Bromopalmitate
26S	26S Proteasome
ACC	Acetyl CoA Carboxylase
ACLY	ATP Citrate Lyase
ADAM	A Disintegrin and Metalloproteinase Domain-Containing Protein
AHA	Azidohomoalanine
APT	Acyl-protein Thioesterase
AR	Androgen Receptor
Az-Palm	15-Azidopentadecanoic Acid/Azido-palmitate
BF	Brefeldin A
BMI	Body Mass Index
BPH	Benign Prostatic Hyperplasia
CPT-1	Carnitine Palmitoyltransferase-1
CX	Cycloheximide
DMSO	Dimethyl Sulfoxide
EEA1	Early Endosome Antigen 1
ER	Endoplasmic Reticulum
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition

eNOS	Endothelial Nitric Oxide Synthase
FASN	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FPP	Farnesyl Pyrophosphate
FTI	Farnesyltransferase Inhibitor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGPP	Geranylgeranyl Pyrophosphate
GGTI	Geranylgeranyltransferase inhibitor
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia-Inducible Factor 1α
HRP	Horseradish Peroxidase
HSP90	Heat Shock Protein 90
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Immunoprecipitation
iPrEC	Immortalized Human Prostate Epithelial Cells
MBOAT	Membrane-bound O-acyltransferase
MET (gene)	Mesenchymal to Epithelial Transition
MN	Monensin
mTOR	Mammalian Target of Rapamycin
NMT	N-Myristoyltransferase
ODYA	17-Octadecynoic Acid
OH-MYR	Hydroxymyristate

PAGE	Polyacrylamide Gel Electrophoresis
PAT	Palmitoyl Acyltransferase
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-Kinase
PIN	Prostatic Intraepithelial Neoplasia
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PUFA	Polyunsaturated Fatty Acids
PTEN	Phosphatase and Tensin Homolog
RTK	Receptor Tyrosine Kinase
SREBP-1	Sterol Regulatory Element Binding Protein-1
SV	Secretory Vesicles
T_0	Time Zero
TRAMP	Transgenic Adenocarcinoma Mouse Prostate
UTR	Untranslated Region
VEGFR	Vascular Endothelial Growth Factor Receptor

Manuscripts

Coleman, D.T., Bigelow, B., and Cardelli, J. Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Mol. Cancer. Ther. 8(1): 214-224.

Milligan, S.A., Burke, P., Steffan, J.J., **Coleman**, D.T., Bigelow, R.L., Carroll. J.L., Williams, B.J., and Cardelli, J. The Green Tea Polyphenol, EGCG, Potentiates the Anti-Proliferative Activity of c-met and EGFR Inhibitors in Non-Small Cell Lung Cancer Cells. Clin. Cancer Res. 2009 Aug 1; 15(5): 4885-4894.

Duhon D., Bigelow R.L., **Coleman** D.T., Steffan J.J., Yu, C., Langston W., Kevil C.G., and Cardelli J.A. The Polyphenol Epigallocatechin-3-Gallate Affects Lipid Rafts to Block Activation of the c-Met Receptor in Prostate Cancer Cells. Mol. Carcinogenesis. 2010 June 1; 49: 739-749.

*Steffan, J.J., ***Coleman**, D.T., Cardelli, J. A. Review: The HGF-Met Signaling Axis: Emerging Themes and Targets of Inhibition. Current Protein & Peptide Science. 2011 Feb. 1, 12(1).

Coleman, D.T. and J. A. Cardelli. Book Chapter: Polyphenols as Receptor Tyrosine Kinase Inhibitors and Anti-Cancer Agents. Springer. Nutraceuticals and Cancer. 2011 Oct. 1.

Cardelli, J., **Coleman, D.T**., Crew, D.K, Book Chapter: Flavonoids: Impact on Prostate Cancer and Breast Cancer. Springer. Pharmaceutical Research. Submitted 2012.

Coleman, D.T., Gray, A., Cardelli, J.A. Palmitoylation Regulates the Trafficking and Stability of c-Met. In preparation.

Coleman, D.T.*, Song, Y.*, Cardelli, J.A., Chung, Jun. Inhibition of Integrin β4 Palmitoylation by Curcumin Prevents Lipid Raft Redistribution and Breast Cancer Cell Motility. In preparation.

Sakr, H., **Coleman, D.T**., Bhatia, S., Cardelli, J.A., Mathis, M. Generating a Replication Competent Adenovirus Vector that Targets the c-Met Receptor. In preparation.

Published Abstracts

Coleman, D.T., and Cardelli, J. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels. Ray A. Barlow Scientific Symposium on Natural Products as Anti-Cancer Agents. Abstract 4, April 2008.

Coleman, D.T., and Cardelli, J. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels. Graduate Student Research Day, LSU Health Sciences Center, Shreveport, LA. May 2008.

Coleman, D.T., and Cardelli, J. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels. Proceedings of the American Association of Cancer Research, Abstract 4655, April 2008.

Coleman, D.T., and Cardelli, J. Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Joint Metastasis Research Society – AACR Conference on Metastasis, Abstract A26, August 2008.

Coleman, D.T., and Cardelli, J. Inhibition of Fatty Acid Synthase Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Ray A. Barlow Scientific Symposium on Inflammation and Cancer. Abstract. April 2009.

Coleman, D.T., and Cardelli, J. Inhibition of Fatty Acid Synthase Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Graduate Student Research Day, LSU Health Sciences Center, Shreveport, LA. May 2009.

Coleman, D.T., and Cardelli, J. c-Met Protein Expression is Regulated by Palmitoylation in Prostate Cancer Cells. Proceedings of the American Association of Cancer Research, Abstract 7446, April 2012.

Coleman, D.T., and Cardelli, J. Regulation of c-Met by fatty acid synthase activity and palmitoylation. Proceedings of the American Association of Cancer Research, Abstract 247210_1, January 2013.

Coleman, **D.T**., Bigelow, B., and Cardelli, J.A. Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Mol. Cancer. Ther. 2009 Jan 1; 8(1): 214-224.

Milligan, S.A., Burke, P., Steffan, J.J., **Coleman, D.T**., Bigelow, R.L., Carroll. J.L., Williams, B.J., and Cardelli, J.A. The Green Tea Polyphenol, EGCG, Potentiates the Anti-Proliferative Activity of c-met and EGFR Inhibitors in Non-Small Cell Lung Cancer Cells. Clin. Cancer Res. 2009 Aug 1; 15(5): 4885-4894.

Duhon D., Bigelow R.L., **Coleman D.T**., Steffan J.J., Yu, C., Langston W., Kevil C.G., and Cardelli J.A. The Polyphenol Epigallocatechin-3-Gallate Affects Lipid Rafts to Block Activation of the c-Met Receptor in Prostate Cancer Cells. Mol. Carcinogenesis. 2010 June 1; 49(8): 739-749.

***Coleman, D.T**., *Steffan, J.J., and Cardelli, J. A. Review: The HGF-Met Signaling Axis: Emerging Themes and Targets of Inhibition. Current Protein & Peptide Science. 2011 Feb. 1, 12(1): 12-22. *Authors contributed equally to study.

Coleman, D.T. and J. A. Cardelli. Book Chapter: Polyphenols as Receptor Tyrosine Kinase Inhibitors and Anti-Cancer Agents. Springer. Nutraceuticals and Cancer. 2012 Oct. 1: 39-60.

Cardelli, J.A., **Coleman, D.T**., and Crew, D.K, Book Chapter: Flavonoids: Impact on Prostate Cancer and Breast Cancer. Springer. Pharmaceutical Research. Submitted 2012.

Sakr, H., **Coleman, D.T**., Bhatia, S., Cardelli, J.A., and Mathis, M. Generating a Replication Competent Adenovirus Vector that Targets the c-Met Receptor. PLoS ONE. Submitted 2013.

Steffan, J.J., Dykes, S.S., **Coleman, D.T**, Adams, L.K., Rogers, D., Carroll, J.L., Williams, B.J., and Cardelli, J.A. A Potential Role for the GTPase Rab7 in Prostate Cancer Progression**,** Scientific Reports. Submitted 2013.

Coleman, D.T., Gray, A.L., and Cardelli, J.A. Palmitoylation Regulates the Trafficking and Stability of the Receptor Tyrosine Kinase c-Met. In preparation.

***Coleman, D.T.,** *Song, Y., Cardelli, J.A., and Chung, J. Inhibition of Integrin β4 Palmitoylation by Curcumin Prevents Lipid Raft Redistribution and Breast Cancer Cell Motility. In preparation. *Authors contributed equally to study.

Gray, A.L., **Coleman, D.T**., and Cardelli, J.A. MCT1 is a Translational Regulator of the c-Met Receptor Tyrosine Kinase. In preparation**.**

Published Abstracts

Coleman, D.T., and Cardelli, J.A. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels.

Ray A. Barlow Scientific Symposium on Natural Products as Anti-Cancer Agents. Abstract 4, April 2008.

Coleman, D.T., and Cardelli, J.A. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels. Graduate Student Research Day, LSU Health Sciences Center, Shreveport, LA. May 2008.

Coleman, D.T., and Cardelli, J.A. The Polyphenol Luteolin Inhibits HGF-Induced Scattering and Motility Concurrent with Post-Translationally Lowering c-Met Levels. Proceedings of the American Association of Cancer Research, Abstract 4655, April 2008.

Coleman, D.T., and Cardelli, J.A. Inhibition of Fatty Acid Synthase by Luteolin Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Joint Metastasis Research Society –American Association of Cancer Research Conference on Metastasis, Abstract A26, August 2008.

Coleman, D.T., and Cardelli, J.A. Inhibition of Fatty Acid Synthase Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Ray A. Barlow Scientific Symposium on Inflammation and Cancer. Abstract. April 2009.

Coleman, D.T., and Cardelli, J.A. Inhibition of Fatty Acid Synthase Post-Transcriptionally Downregulates c-Met Expression Independent of Proteosomal/Lysosomal Degradation. Graduate Student Research Day, LSU Health Sciences Center, Shreveport, LA. May 2009.

Coleman, D.T., and Cardelli, J.A. c-Met Protein Expression is Regulated by Palmitoylation in Prostate Cancer Cells. Proceedings of the American Association of Cancer Research, Abstract 7446, April 2012.

Coleman, D.T., and Cardelli, J.A. Regulation of c-Met by fatty acid synthase activity and palmitoylation. Proceedings of the American Association of Cancer Research, Abstract 247210_1, January 2013.